VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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Preface

The Editors take pleasure in presenting this eighteenth volume of Vitamins and Hormones

An unusual departure from previous policy is the inclusion of the papers delivered at the Symposium on Vitamin A and Metabolism, held in honor of Professor P Karrer at Burgenstock, Switzerland, on May 23, 24, and 25 1960 This does not represent a permanent change in editorial policy, although it is not impossible that it might be repeated if another symposium so specifically in our field of interest and of equal breadth and importance were to take place Rather it should be considered as an experiment, on the results of which future policy may depend The editors would be pleased to receive readers' comments upon it It had been hoped that this symposium could be published within a few months of its occurrence by including it herewith without delaying the appearance of the volume We regret that this has proved impossible

Volume 18 contains four chapters on vitamins and three on hormones, in addition to those in the Vitamin A Symposium All of these chapters are critical reviews in which completeness of coverage has not been allowed to swamp that personal approach which makes a chapter readable We feel that they are important contributions to understanding in this field

In his remarks (p 571) at the close of the Vitamin A Symposium, Professor Karrer quotes Goethe as saying "Science is a great musical fugue, in which the voice of every nation finds its expression," and goes on to ask "Is there anything better than these free discussions from country to country from continent to continent which do not serve to subject humanity nor impose power, but which are aimed at unveiling the secrets of nature and extending our understanding of the universe?" These thoughts are, it seems to us particularly appropriate for Vitamins and Hormones since this publication is international in authorship, supranational in subject matter and world wide in distribution

The Editors wish to express warm appreciation to the 29 authors from five countries who have contributed to Volume 18 It is our eager hope that they will feel a deep satisfaction in having performed this important service for their colleagues

ROBERT S HARRIS
DWIGHT J INGLE

December 1960

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Physiology and Biochemistry of Biotin

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I Introduction

The aim of this chapter is to discuss the principal characteristics of the physiology and biochemistry of biotin We have considered not only new data which have appeared since the review by Lichstein (1951), but also recalled earlier facts which did not come within the compass of that author's review

The present work is not complete In particular, we have not considered the problems of the specificity of action of biotin, the role of biotin in resistance to infectious diseases, and the improbable connection between

biotin and cancer These questions have been dealt with in detail by Gyorgy (1954)

In considering the metabolic activities of biotin, a parallel has been established, whenever possible, between the participation of the vitamin in microbial metabolism and in animal metabolism In this way it is possible to show clearly the universal or limited ability of biotin to participate in certain metabolic operations, to demonstrate the common or separate processes by which biotin intervenes in these operations in microorganisms and in animals, and to point out the gaps relative to the possible implication of biotin in these two metabolisms

Certain other aspects of the action of biotin come solely within the compass of animal physiology, e g the problems of the relationship between biotin and the endocrine glands, the nervous system and the skin Although they are of limited bearing, it is essential to understand these relationships in order to be able to define the properties of biotin

II The Metabolic Activities of Biotin

1 Carbohydrate Metabolism

Biotin takes part in the following reactions (1) the Wood Werkman reaction proceeding to the reversible conversion of pyruvic acid to oxalacetic acid and vice versa, (2) conversion of malic acid to pyruvic acid in the presence of malic enzyme, (3) interconversion of succinic acid and propionic acid, (4) conversion of oxalosuccinic acid to α ketoglutaric acid, (5) different enzymatic reactions of dehydrogenation, such as that of succinic acid For the sake of clarity these reactions have been grouped with carbohydrate metabolism, but it is obvious that several of them have a much wider implication and participate in the intermediate metabolism of ternary chains in general

The first four reactions are carboxylation and decarboxylation reactions This fact is of prime importance Indeed, while certain biochemical actions of biotin are still obscure, it seems incontestable that this vitamin participates in many carboxylation and decarboxylation reactions not only in carbohydrate metabolism, but also as will be seen, in lipid, protein and nucleic acid metabolism

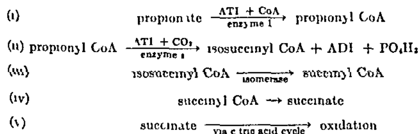
For some of the above five reactions, the participation of biotin has been demonstrated until now only in microorganisms, for others, the vitamin plays a general role, since it has also been recognized in the tissues of mammals and birds Among the latter reactions, that of Wood Werkman is of fundamental importance By its participation in this reaction, biotin—along with thiamine, riboflavin, niacinamide, and pantothenic acid—plays an indispensable role in the oxidation cycle of the ter

nary chains This role is specifically to provide the oxalacetic acid for this cycle

The participation of biotin in the reactions of carbohydrate metabolism enumerated above has been thoroughly discussed by Lichstein (1951) We mention here only the new facts, indeed few in number, which have appeared since 1951

a Probable Coenzyme Nature of Biotin in Oxalacetate Decarboxylase It seems probable that biotin participates as a coferment in the five reactions mentioned above But this presumption will be established only with the isolation of biotin from absolutely pure enzymes The only research carried out in this field has been concerned with the Wood Werkman reaction Lichstein (1955, 1957) succeeded in establishing a very significant correlation between the degree of purity of oxalacetate decarboxylase extracted from the liver of the chick, and the quantity of biotin which is freed by hydrolysis The purest enzyme preparation contains 3 mμg biotin per milligram protein, which represents approximately 1 mole biotin per 10^5 g of protein (Lichstein 1957) However Lichstein's own opinion is that these results are only indicative and that the purification and analysis of the constitution of oxalacetate decarboxylase must be carried out before it can be stated definitely that biotin is its coenzyme

b Interconversion of Succinic Acid to Propionic Acid *i Conversion of propionic acid to succinic acid* (1) Animal metabolism The principal stages of the enzymatic oxidation of propionic acid in animal tissues have been established largely by the work of Ochoa (Flavin and Ochoa, 1957, Flavin *et al*, 1957, Beck *et al* 1957) and Lardy (Lardy and Peanasky, 1953, Lardy and Adler, 1956) as follows



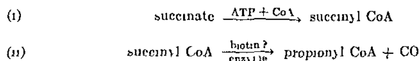
Biotin deficiency decreases the capacity of rat liver mitochondria extract to synthesize succinate from propionate and from $NaHC^{14}O_3$ This capacity is in no way restored by the *in vitro* addition of biotin or biocytin, on the other hand, it reappears very rapidly after administration of biotin to the living deficient animal (Lardy and Adler, 1956)

The precise stage of the intervention of biotin in the conversion of propionic acid to succinic acid still remains to be determined, however This determination is all the more delicate because the mechanism of re

action (ii) in particular is still obscure. This reaction is probably catalyzed by a single enzyme which is referred to as a propionyl carboxylase. This purified enzyme contains no biotin (Tietz and Ochoa, 1959). Thus, the reported function of biotin in propionate carboxylation remains unexplained.

(2) Metabolism of microorganisms. *Propionibacterium pentosaceum* (Barban and Ajl, 1951; Delwiche *et al.*, 1953) and *Micrococcus lactilyticus* (Whiteley, 1953b) also convert propionic acid to succinic acid, but it has not been possible to determine whether the mechanism is the same as in animal tissues.

ii. Conversion of succinic acid to propionic acid. Metabolism of microorganisms. Since the initial work of Delwiche (1950) and of Lichstein (1950) related by the latter in 1951, supplementary information has been published on the decarboxylation of succinic acid to propionic acid and the part that biotin plays in it. This reaction, according to Whiteley (1953a), is the principal pathway for the formation of propionic acid in *P. pentosaceum*, *M. lactilyticus*, and other micrococci. The conversion of succinic acid to propionic acid consists of the following two enzymatic operations (Delwiche *et al.*, 1953; Whiteley, 1953a,b; Chambers and Delwiche, 1954):



(i) $$\text{succinate} \xrightarrow{\text{ATP + CoA}} \text{succinyl CoA}$$

(ii) $$\text{succinyl CoA} \xrightarrow[\text{enzyme}]{\text{biotin ?}} \text{propionyl CoA} + CO$$

But no mention is made of the stage of the isosuccinyl CoA step or of the presence of adenosine triphosphate (ATP) in phase (ii) of the reaction (Whiteley, 1953a,b). It is therefore not yet possible to decide whether these two observations are sufficient to establish the real differences between bacterial metabolism and animal metabolism in the interconversion of succinic acid to propionic acid. If the reaction converting succinic acid to propionic acid in microorganisms seems less complex than the one converting propionic acid to succinic acid in higher animals, it may be simply because it has not been as closely studied.

Seeking to determine exactly the site of biotin involvement in the conversion by microorganisms of succinic acid to propionic acid, Chambers and Delwiche (1954) showed that the vitamin does not participate in the first stage of the reaction. It would then seem that biotin is not involved either directly or indirectly in the formation of succinyl coenzyme A. It must, therefore, be involved in the second phase of the reaction, but its exact role has not yet been defined. Does it participate directly in the formation of the apoenzyme or the coenzyme of the succinyl CoA decarboxylase, or indirectly in the synthesis of the protein fraction of this

enzyme? These hypotheses were proposed by Chambers and Delwiche (1954)

Thus, the precise role of biotin in succinic acid propionic acid interconversion is not known, either in animals or in microorganisms

Lichstein (1958) stresses that biotin deficiency decreases the ability of *P pentosaceum* both to decarboxylate succinic acid to propionic acid and to ferment glucose Under these conditions, the glucose fermentation is restored by adding either biotin or desthiobiotin or oxybiotin to the medium, but the latter derivative stimulates the decarboxylation of succinic acid only weakly compared with biotin or desthiobiotin, which are equally effective It might be that oxybiotin in particular is converted to an oxybiotin coenzyme which has relatively poor ability to serve as a cofactor for the succinate decarboxylase system but is fully capable of replacing the biotin coenzyme in the fermentation of glucose These different considerations led Lichstein (1958) to the very interesting suggestion that there might be several coenzyme forms of biotin in the same way that there are several coenzyme forms of niacinamide and pyridoxine

c Hexokinase A culture of *Leuconostoc mesenteroides* which does not require biotin when its medium contains saccharose needs this vitamin if the medium contains a monosaccharide (Carlson and Whiteside Carlson 1949) In the same way, a biotin-deprived culture of *Saccharomyces cerevisiae* (1939 strain) continues to grow moderately if the medium contains saccharose but does not grow at all if it contains glucose (Williams *et al*, 1957)

On the basis of these observations, Williams *et al* (1957) proposed that biotin may control the initial stage of glucose catabolism, i e phosphorylation catalyzed by hexokinase (Fig 1)

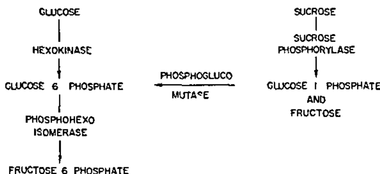


Fig 1 Initial steps in the catabolism of glucose and sucrose According to Williams *et al* (1957)

The authors confirmed their hypothesis by showing that the phosphorylation velocity of the 2 deoxy D glucose is greatly reduced in a cell free

extract of *S cerevisiae* deficient in biotin Strauss and Moat (1958) give the following supplementary information glucose 6 phosphate or fructose 6 phosphate fermentation, is independent of biotin in the same way as hexose diphosphate, the activity of phosphoglucoisomerase and that of glucose 6 phosphate dehydrogenase are also independent of biotin

These facts taken together indicate that there is a close relationship between biotin and hexokinase The hexokinase reaction involves the dissociation of a proton from a hydroxyl group of glucose before phosphorylation Possibly in the enzymatic systems in which biotin plays a part, it might, according to the hypothesis of Lichstein (1951), play a unique role in the hydrogen transport involved in these reactions The mode of action of biotin might be, in the case of hexokinase, as Strauss and Moat suggested, to aid the dissociation of a hydrogen atom from an otherwise undissociable hydroxyl group of glucose Strauss and Moat (1958) have gone still further in determining the link between biotin and hexokinase activity and have shown that the addition of free biotin *in vitro* restores the weakened hexokinase activity of biotin deficient yeast extract cells under different conditions This is, moreover, an exceptional observation in the biochemistry of biotin for most of the other enzymatic reactions that are weakened by biotin deficiency cannot be stimulated by the *in vitro* addition of this vitamin It must be pointed out that it is this fact which casts doubt on the coenzyme nature of biotin in these enzymatic reactions

The functional relationship between biotin and hexokinase shown above in *S cerevisiae* strain 139 has never been observed in *P pentosaceum*, strain E 214, although biotin deficiency reduces the ability of this microorganism to ferment glucose (Lichstein, 1958) In a mutant strain of *Escherichia coli* (23358) this functional relationship does not appear either (Ferguson and Lichstein, 1957) On the contrary, the presence of glucose triples or quintuples the growth of *E coli* 23358 when this microorganism is deficient or partially deficient in biotin The mechanism of this stimulating action is still unknown, it cannot be explained by a synthesis of biotin since the content of this vitamin in this microorganism stays the same in the presence or absence of glucose

The role of biotin in the phosphorylation of glucose in yeast perhaps conforms with a more general observation of Sytinskaja (1956) that oxidative phosphorylation is reduced in the liver extracts of biotin deficient rats The administration of the vitamin only 90 minutes prior to killing restores phosphorylation to normal

d In Vivo Carbohydrate Metabolism of the Rat i Pyruvic acid level in blood It is undeniable that biotin deficiency produces an important increase in blood pyruvic acid T Terroine (1956a) observed it to be dou

bled This increased blood pyruvic acid is easily conceived as the direct result of the slowing-down, because of biotin deficiency, of the conversion of pyruvic acid to oxalacetic acid What is worth noticing, for it is seldom encountered, is that these results *in vivo* correspond perfectly with the results *in vitro*, showing a poor utilization of pyruvic acid by biotin-deficient rat liver slices or homogenates (Summerson *et al*, 1941, Pilgrim *et al*, 1942) or by heart ventricle slices from biotin-deficient ducks (Olson *et al*, 1948)

ii Glycemia level It is not illogical to suppose, at first sight that hyperpyruvicemia might be accompanied by a hyperglycemia, the unused glucose accumulating in the general circulation as a result of some disorder at one of the stages of its degradation Though hyperglycemia and hyperpyruvicemia do indeed go together in thiamine deficiency (Lu, 1939 Ferrari, 1953, Di Stefano and Privitera, 1955), rats deprived of biotin do not show any hyperglycemia but on the contrary, a slight hypoglycemia (T Terroine, 1956a)

iii Glucose tolerance test The glycemia of biotin deficient rats, pair fed and ad libitum controls has been studied after intraperitoneal injection of 100 mg glucose per 100 g body weight The latter group showed the most hyperglycemic response, the first group the least The significance and intimate mechanism of the difference in behavior of the three groups is not known (T Terroine, 1956a)

iv Liver and muscle glycogen level Biotin deficiency in no way modifies the glycogen content of liver or muscle (T Terroine 1956a) By the maintenance of a normal liver and muscle glycogen level, biotin deficiency is distinctly different from other vitamin deficiencies Indeed deficiencies in pantothenic acid (Hurley and Mackenzie 1954), in riboflavin (Forker and Fay Morgan, 1954) or in ascorbic acid (Sebrell and Harris, 1954) lower the glycogen concentration of liver and muscle

v Liver and muscle, free reducing sugar level The free reducing sugar level of the liver is considerably lowered in biotin deficiency (T Terroine, 1956a) The intimate cause of this anomaly is still unknown It is difficult to decide whether it can be attributed to a disorder of glycogenolysis due to a slowing down of amylase activity According to *in vitro* studies, the plasma or pancreas amylase activity of the rat is independent of biotin (Ramachandran and Sarma 1954), on the other hand, biotin deficiency appears to reduce considerably the synthesis of amylase by chick pancreas slices (Poznanskaja, 1957) The free reducing sugar level of muscle is in other respects absolutely constant

Acute biotin deficiency in the rat causes hyperpyruvicemia and only minimal additional disorders of carbohydrate metabolism

2 Lipid Metabolism

a Interrelationship between Biotin and Certain Fatty Acids *i Evidence of the vicarious activity of oleic acid and C_{18} polyunsaturated acids toward biotin* (1) Microorganisms Since the initial work of Williams *et al*, related by Lichstein (1951), showing the vicarious role of oleic acid for biotin deficient *Lactobacillus casei*, research has been carried out particularly on the nature of the relationship between fatty acids and the vitamin

The vicarious ability of oleic acid toward biotin extends to numerous microorganisms, such as *L arabinosus* (Axelrod *et al*, 1948, Broquist and Snell, 1951, Cheng *et al*, 1951, Melnick and Deuel, 1954), *Leuconostoc mesenteroides* (Carlson *et al*, 1950), *Clostridium butyricum*, *Streptococcus faecalis* (Broquist and Snell, 1951) *Lactobacillus fermenti* (Broquist and Snell, 1951), numerous strains of *Staphylococcus* (Gretler *et al*, 1955), *Escherichia coli* (Ferguson and Lichstein, 1957), etc

In seeking to determine the structural elements of the oleic acid molecule that specifically confer this vicarious power, it has been possible, in the studies on microorganisms to make the following four important observations (a) The presence of the carboxyl group is necessary, its substitution by an alcohol group stops all vicarious activity for *L arabinosus* (Axelrod *et al*, 1948) (b) The vicarious power is extended to the *cis* isomers of oleic acid (Cheng *et al*, 1951, Melnick and Deuel, 1954) (c) The vicarious power is partially or totally suppressed in the *trans* isomers of oleic acid Elaidic acid may be an exception among these *trans* isomers for it is as active as oleic acid in replacing biotin, according to Cheng *et al* (1951) and Melnick and Deuel (1954) However, Axelrod *et al* (1948) reported that it has no more than one sixth the activity of oleic acid For all the other *trans* isomers of oleic acid, the ability to replace biotin is either weak or nonexistent It is all the more attenuated as the double bond gets further from position 9=10 and nearer the end of the carbon chain In any case, the addition of biotin to a culture medium of *L arabinosus* enables the microorganisms to use the previously inactive *trans* acids This would suggest that biotin may be involved either in the conversion of the *trans* acids to *cis* acids, or in increasing the absorption of the *trans* acids by microorganisms (Cheng *et al*, 1951, Melnick and Deuel, 1954) (d) Polyunsaturated C_{18} fatty acids can also replace biotin Linoleic and linolenic acids, previously detoxified, possess more or less the same vicarious activity as oleic acid for *Clostridium butyricum* or *S faecalis* (Broquist and Snell, 1951), with *L arabinosus*, however, the power of replacing biotin seems to decrease with the number of double bonds (Axelrod *et al*, 1948, Cheng *et al*, 1951)

(2) Animals It is very doubtful that oleic acid can replace biotin in

animals As a matter of fact, the larvae of the mosquito *Aedes aegypti*, can develop in a medium very low in biotin but containing oleic acid (Trager, 1948b), however, the vicarious effect of oleic acid in the biotin deficient chick or rat is far from conclusive

In fact Trager (1948a) found that the fat soluble fraction of the plasma, which Axelrod *et al* (1948) have shown is composed of oleic, linoleic, arachidonic acids and saturated fatty acids when injected into the chick reduced the severity of the deficiency in biotin caused by an egg white diet However, the injection of oleic acid or the oral administration of the fat soluble fraction of the plasma had no effect on this deficiency (Trager, 1948a) The chemical nature of the biotin like substance of plasma has not yet been determined

Also, the presence of oleic acid in a biotin free diet did not increase egg production, hatchability, or the biotin content of egg yolks and whites (Couch *et al*, 1949)

Finally, the supplementation of a biotin deficient dried raw egg white diet with C_{18} unsaturated fatty acids had no beneficial effects on deprived rats according to Nielsen and Elvehjem (1942) and Johnson *et al* (1952) but improved very considerably the general state of these rats according to MacKay and Barnes (1941) However the addition of olive or corn oil to a biotin deficient diet containing no egg white caused a more severe deficiency in coprophagy prevented rats than does hydrogenated coconut oil The differences between the effects of the fats used in this study must probably reflect some metabolic phenomenon in the tissues (Barnes *et al*, 1959)

ii The mechanisms of the vicarious activity of oleic acid toward biotin Three mechanisms have been proposed to explain the vicarious power of C_{18} unsaturated fatty acids, they concern the relationship between oleic acid and biotin The first two explanations suggested by Williams and his collaborators are discussed by Lichstein (1951) in his review

(1) Contribution of biotin to the synthesis of oleic acid If biotin contributes either directly or indirectly to the synthesis of oleic acid it would seem that when the latter is supplied preformed, the vitamin is dispensable or the requirement for biotin is reduced Based on analogy with the aspartic acid biotin relationship this theory has not been founded on any experimental data until now Possibly it is beginning to find support in the field of plant physiology When embryos of the fruit *Linum usitatissimum* L are cultured *in vitro* on an artificial medium containing sodium 1 C^{14} acetate and quantities of biotin varying from 0 to 1000 μg per liter of medium, the presence of biotin in the medium favors the synthesis by the embryos of at least two unsaturated fatty acids, probably linoleic and linolenic acids and it probably enhances the synthesis of oleic acid (Kurtz and Miramon, 1958)

(2) Contribution of biotin and oleic acid to cell permeability. The biological role of biotin could be that of a cell permeability factor increasing the uptake of nutrients by cells. Oleic acid, and certain detergents possessing this same property, could thus replace biotin. The technical difficulties involved in the experimental approach to cell permeability and to surface activity explain that the experimental data, all concerning microorganisms, are not sufficiently extensive or conclusive, in the opinion of Hofmann and Panos (1954).

(3) Contribution of oleic acid to the penetration of biotin into the cell. The cells of *L. arabinosus* are relatively impermeable to biotin. Oleic acid, and perhaps other unsaturated fatty acids, could permit a better penetration of biotin into bacterial cells and would thus exert a sparing effect on the biotin requirements of this microorganism. If this be so, oleic acid cannot exercise its vicarious activity in the complete absence of biotin (Traub and Lichstein, 1956). This was also the opinion of Broquist and Snell (1953), although their interpretation of the facts differs from that of Traub and Lichstein. Broquist and Snell (1951) observed that the cells of *L. arabinosus* growing on a medium deprived of biotin but containing oleic acid nevertheless still contain some traces of the vitamin, probably synthetized by the microorganism. The vicarious activity of oleic acid toward biotin in *L. arabinosus* is abolished in the presence of avidin (Broquist and Snell, 1953). The authors think that the avidin probably combines with the traces of biotin contained in the microorganism, thus making it unavailable. This proves that this minute quantity of biotin is indispensable for growth of *L. arabinosus*, even in the presence of oleic acid (Broquist and Snell, 1953). To Traub and Lichstein (1956), the role of oleic acid is to permit the entry of this minute quantity of indispensable biotin into the cell, as has been shown by the work of Broquist and Snell (1951, 1953).

Lichstein and Ferguson (1958) bring new information concerning the conditions that govern the entrance of biotin into cells of *L. arabinosus*. This entrance requires energy, is greatly increased by the presence of glucose, the favorable action of which is inhibited by iodoacetate, and is completely abolished in the presence of homobiotin.

As the authors themselves (Traub and Lichstein, 1956) point out, the facts shown only for *L. arabinosus* should be extended to other microorganisms. The observation of Broquist and Snell (1953) seems to mitigate against this theory, for they have observed that the vicarious activity of oleic acid toward biotin is in no way suppressed in the presence of an excess of avidin with *S. faecalis*, *Leuconostoc mesenteroides*, *Lactobacillus delbrueckii*, *L. fermenti*, or *L. casei*. This would mean that oleic acid retains completely its power to stimulate the growth of these micro

organisms, in the absence of biotin Williams and Fieger (1947) have also shown that oleic acid retains all its power to stimulate growth of *L casei* in the presence of avidin, which combines with all the biotin in the medium

Andrews and Williams (1951) observed that washed cells of *L casei* harvested from an oleic acid medium contain ten times as much biotin as the corresponding uninoculated medium It is doubtless tempting bearing in mind the hypothesis of Traub and Lichstein, to suppose that oleic acid facilitates the entrance of biotin into the cells of *L casei* But this suggestion does not conform with the previous observations of Williams and Fieger (1917) and Broquist and Snell (1953) showing the persistence of the activity of oleic acid in stimulating the growth of *L casei* even in the total absence of biotin

iii Vicarious power of long chain saturated fatty acids toward biotin in microorganisms In microorganisms, long chain saturated fatty acids also possess a vicarious activity toward biotin

Indeed, palmitic and stearic acids inactive in themselves, enhance the vicarious power of oleic acid for *L arabinosus* or *Clostridium butyricum* deprived of biotin (Axelrod *et al*, 1948, Broquist and Snell, 1951) They both play a sparing role toward oleic acid, itself a sparing factor of biotin

The mechanism of the favorable influence of saturated fatty acids is not known They contribute in detoxifying unsaturated fatty acids, but this cannot explain this phenomenon Indeed palmitic and stearic acids retain their enhancing capacity toward oleic acid whether this acid is used in detoxified form (Broquist and Snell 1951) or not (Axelrod *et al*, 1948)

Other saturated fatty acids may like oleic acid and the C_{18} polyunsaturated acids, replace biotin in the nutrition of numerous microorganisms

Such is the case for lactobacillic acid one of the most important constituents of the lipids of *L arabinosus* and of *L casei* Thus, lactobacillic acid can be substituted for biotin in the nutrition of *L casei*, *L arabinosus*, and *L delbrueckii* Isomers and related substances of lactobacillic acid also have this power The vicarious power of these saturated cyclopropane ring fatty acids depends on the chain length If it is less than 11 carbon atoms, not only does the fatty acid lose its vicarious power toward biotin but it may even exert an inhibitory action for certain bacilli In the absence of biotin some microorganisms, such as *L casei*, grow as well with C_{18} unsaturated acids as with cyclopropane ring saturated fatty acids, others such as *L arabinosus*, do much better with the former than with the latter (Hofmann and Panos, 1954)

Another saturated fatty acid, lauric acid, a C_{12} acid, enhances the growth of *Bacillus coli* in the presence of a suboptimum quantity of biotin, but

not in its complete absence (Ferguson and Lichstein, 1957). When added to the medium in form of Tween 20, lauric acid can also replace biotin for *Leuconostoc mesenteroides* (Carlson *et al.*, 1950).

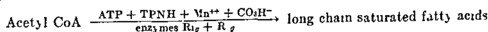
The mechanism of the vicarious activity of all these saturated fatty acids is unknown.

b. General Processes of Lipid Metabolism. Here are grouped results of research dealing with various fields of the physiology of lipids.

i. Absorption. Biotin deficiency does not seem to modify the absorption of olive oil. After a 24 hour fast, biotin deficient rats received 1 *ml* olive oil and 6½ hours later its distribution in the digestive tract was measured. The olive oil left the stomach much more quickly than in the controls. Moreover, the increased intestinal motility of the deficient rats in no way modified the absorption of the oil in this portion of the digestive tract (Woodruff, 1952).

ii. Fatty acid, glyceride, and phosphatide synthesis. (1) Fatty acids. The data concerning the participation of biotin in the synthesis of fatty acids are contradictory.

The total fatty acid content of rat liver triples when the animals receive a diet containing an excess of cholesterol. This accumulation of fatty acids does not occur when this excess of cholesterol is administered to rats deficient in biotin. Okey *et al.* (1951) suggested that biotin is necessary to the synthesis and storage of fatty acids. In fact, two groups of *in vitro* studies show clearly that biotin is implicated in the synthesis of at least certain fatty acids. First, there is the observation mentioned above of the participation of biotin in the synthesis of C_{18} unsaturated fatty acids by *Linum usitatissimum* embryos (Kurtz and Miramon, 1958). Then, there is the study carried out with highly purified pigeon liver extracts, involving biotin in a new enzymatic system for the synthesis of long chain fatty acids from acetyl CoA (Wakil *et al.*, 1958). This synthesis also requires the presence of ATP, TPNH (reduced triphosphopyridine nucleotide), the ions Mn^{++} and CO_3H^-, and finally two highly purified enzymes R_{1g} and R_{2g}. Schematically this synthesis reaction can be represented as follows:



$$\text{Acetyl CoA} \xrightarrow[\text{enzymes } R_{1g} + R_{2g}]{\text{ATP} + \text{TPNH} + Mn^{++} + CO_3H^-} \text{long chain saturated fatty acids}$$

Among the saturated fatty acids synthesized this way are palmitic acid and smaller quantities of myristic, lauric, and decanoic acids. Butyric acid or hexanoic acid do not appear to be synthesized or incorporated in the longer chain acids (Gibson *et al.*, 1958).

Enzyme R_{1g} contains a very high proportion of biotin: between 200 and 250 mμg. per milligram of protein, or about 1 mole biotin per $1 \cdot 10^6$ g. protein. This is the highest ratio between biotin and protein yet re

ported (Wakil *et al*, 1958) Support for the implication of biotin in this synthesis reaction comes from the fact that the conversion of acetyl CoA to palmitate is inhibited in the presence of avidin This inhibition is removed by the introduction of a supplement of biotin (Wakil *et al*, 1958) Thus, once again, biotin appears to intervene, by a mechanism which still remains entirely unexplained in a reaction in which the presence of CO_2 is indispensable (Gibson *et al*, 1958)

The above results do not agree with the following observations The total fatty acid level is more or less identical in the liver, heart, and blood of biotin deficient rats in comparison with pair fed control and ad libitum control groups (Guggenheim and Olson 1952) Also, on the basis of research using either deuterium (Curran, 1950) or acetate *1* C^{14} (Guggenheim and Olson, 1952), the incorporation rate of these substances reveals a normal capacity of biotin deficient rats to synthesize fatty acids The contribution of biotin to fatty acid synthesis as shown by the *in vitro* studies, does not therefore appear to influence the total amount of fatty acids in the living deficient rat

(2) Glycerides and phosphatides After comparing the incorporation rate of acetate *2* C^{14} by biotin deficient rats and pair weighed controls Gram and Okey (1958) made the following three observations (a) The deficient animals excrete the greater part of the acetate *2* C^{14} in the form of $C^{14}O_2$ (b) Acetate *2* C^{14} is only weakly incorporated into liver lipids (c) On the other hand, preference is shown for the use of labeled acetate in the synthesis of glycogen From these results taken together, the authors think it may be deduced that there is an inhibition in the synthesis of glycerides and phosphatides in biotin deficiency As a working hypothesis they suggest that in the absence of biotin this inhibition may depend on a decrease in the synthesis of certain fatty acids This hypothesis is all the more plausible since as has been mentioned, biotin may participate *in vitro*, in the synthesis of certain fatty acids

It would be very interesting to find out whether or not biotin participates in the synthesis of fatty acids glycerides, and phosphatides in microorganisms also

iii Oxidation of fatty acids The dehydrogenation of lauric, myristic, palmitic, and stearic acids is decreased in *L arabinosus* deprived of biotin Under these conditions, the addition of aspartate or oleate to the medium increases this effect In the same way, the activity of dehydrogenase of the fatty acids of *E coli* is decreased in the absence of biotin (Gothoskar and Sreenivasan 1953)

Although Plaut (1951) reported that biotin deficiency does not seem to affect the oxidation of caprylic acid in the rat the results of Rossi *et al* (1957) are more finely drawn A moderate deficiency of biotin does not

modify *in vitro* the oxidation rate of caprylic acid, but an acute deficiency greatly reduces its oxidation rate, and also that of butyric acid The oxidation of lauric acid *in vitro* appears at first to be stimulated by a slight deficiency, then considerably decreased by a serious deficiency, in biotin In respect to these differences of reactivity, it is probable that the enzymes responsible for the oxidation of long chain fatty acids are different from those controlling the oxidation of short chain fatty acids (Rossi *et al*, 1957)

The exact nature of the participation of biotin in these enzymatic reactions has not yet been studied As a hypothesis Gothoskar and Sreenivasan (1953) suggest that biotin may contribute to the synthesis of the amino acids present in the enzymes involved in the oxidation of fatty acids, in certain microorganisms at least

iv Hepatic steatosis The participation of biotin in the appearance of a fatty liver in the rat has involved the use of one of the three following diets (1) high carbohydrate diet deprived of all the B vitamins, used by Gavin and McHenry (1941), MacFarland and McHenry (1945), and Best *et al* (1946), (2) vitamin balanced high carbohydrate diet, also used by Best *et al* (1946) and by Lino (1952), and (3), a protein free diet administered to rats made completely deficient in biotin by previously feeding a dried egg-white diet (T Terroine and Rombauts, 1952)

After the preliminary use of one of the three diets, the administration of an excess of biotin leads to the development of a fatty liver (Gavin and McHenry, 1941, MacFarland and McHenry, 1945, Best *et al*, 1946, Lino, 1952, T Terroine and Rombauts, 1952) It must be pointed out that in the case of the preliminary high carbohydrate diet deprived of B vitamins, the overload of biotin is accompanied by that of the other missing vitamins

Best *et al* (1946), analyzing the formation of fatty liver by the preliminary use of the high carbohydrate diet deprived of all B vitamins, made the following three observations (1) The production of fatty liver by an excess of biotin is not absolutely systematic (2) This production of fatty liver is, as is confirmed by Lino (1952), very weak if the vitamins were balanced during the preliminary period, this would suggest, according to Best *et al* (1946), that the production of fatty liver is a consequence either of the initial state of a multivitamin deficiency or of chronic starvation because the daily ingestion of diet fell rapidly from 10 to 4 g (3) Finally, and above all, the fatty liver produced by an excess of biotin under these special conditions presents no specificity as to the nature of the fatty deposits and the cholesterol ester content, contrary to the results obtained by Gavin and McHenry (1941) and MacFarland and McHenry (1945)

Without contesting the possibility of the appearance of a fatty liver by the administration of an excess of biotin under the conditions indicated above, Best *et al* (1946) disagree with the term "biotin fatty liver," which would imply precisely a specificity of action of biotin in this field

3 Cholesterol Metabolism

The results suggesting a possible participation of biotin in cholesterol metabolism are contradictory and do not permit a clear-cut conclusion Thus some investigators reported that biotin deficiency in the rat has no effect on the synthesis of cholesterol whereas others observed that biotin reduces it

On the basis of rate of incorporation of deuterium in cholesterol, Curran (1950) concluded that biotin is not implicated in cholesterol synthesis Moreover, the cholesterol content of the liver and adrenals was about the same in the biotin-deficient rats and in the pair fed controls (Guggenheim and Olson, 1952)

The positive results involving biotin in cholesterol synthesis are more numerous, but are in disagreement These studies generally consist in determining the part played by biotin in cholesterol synthesis from labeled acetate in biotin deficient rats or in liver slices from these deficient rats

After the administration *in vivo* of acetate *1* C^{14} Guggenheim and Olson (1952) found that in comparison with the pair fed controls, the incorporation of this substance into the cholesterol of the liver and adrenals of deficient rats is considerably reduced, this fact would indicate a reduction of cholesterol synthesis In a complex study, Gram and Okey (1958) also concluded that there is an inhibition of cholesterol synthesis from labeled acetate in the deficient rat compared with the pair weighed control, both because the C^{14} content of the liver was less than that of the pair weighed control and because the greater part of the acetate *2* C^{14} is excreted in the form of $C^{14}O_2$ On the other hand, biotin does not appear to be necessary to the synthesis of cholesterol from acetate, as is shown by the work of Jacobsohn and Corley (1957) who incubated rat liver slices in the presence of acetate *2* C^{14} But Jacobsohn and Corley (1957) observed that biotin deficiency considerably decreased the *in vitro* incorporation of radioactive carboxyl of dimethylacrylic acid into sterol, this would suggest that the use of dimethylacrylic acid for sterol synthesis is favored by the presence of biotin

On the other hand, Okey *et al* (1951) think that biotin deficiency plays only a secondary role in cholesterol storage They showed that when cholesterol was fed to rats receiving a dried raw egg white diet there was a block in the deposition of cholesterol in the liver Barnes *et al* (1959) confirmed this observation under the same experimental conditions But

this interference with cholesterol storage was not observed in rats made mildly deficient on a biotin free diet without avidin in which coprophagy was prevented Therefore, it would appear that the biotin deficiency per se may not be responsible for blocking the storage of cholesterol in the liver and the avidin induced deficiency may have introduced a *factor other* than biotin deficiency that is involved in this storage (Barnes *et al*, 1959)

Biotin deficiency is associated with hypercholesterolemia, as it is observed in man (Sydenstricker *et al*, 1942, Scott, 1959), in rabbit (Curran, unpublished), and in rat (Barnes *et al*, 1959) Might this hypercholesterolemia be due to a disorder in cholesterol storage? Curran (1950) thinks that this is so, and that cholesterol normally synthesized in biotin deficiency, but no longer retained in the liver, passes in excess into the blood

It should be remembered that, *in the previously mentioned conditions* of the appearance of a fatty liver in the rat by an excess of biotin, this liver would appear to be particularly rich in cholesterol not as a result of an increase of its synthesis, but because of an increase of its storage capacity (Gavin and McHenry, 1941) But these results are categorically denied by Best *et al* (1946)

It should be added that, from a study *in vitro*, the cholesterolesterase of dog serum retains the same activity whether or not biotin is present (Del Vecchio and Orengo, 1953)

4 Protein Metabolism

a Metabolism of certain amino acids i Glycine A unique observation is that a large excess of glycine in the diet of rats produces alopecia, disorders of gait, and decrease in growth—disorders in which the administration of biotin seems to exert an immediate beneficial effect (Page and Gingras, 1947)

ii Leucine Biotin deficiency in mammals hinders the normal conversion of the deaminated chain of leucine to acetoacetate carried out by the following series of reactions

leucine $\rightarrow$ α ketoisocaproic acid $\rightleftarrows$ isovaleryl CoA $\rightarrow$

senecyl CoA $\underset{\leftarrow}{\overset{H_2O}{\rightarrow}}$ β hydroxyisovaleryl CoA $\rightarrow$

$\underset{\leftarrow}{\overset{CO_2 + ATP}{\rightarrow}}$ β hydroxy β methylglutaryl CoA $\rightleftarrows$ acetoacetate + acetyl CoA

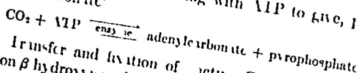
Indeed, in liver homogenate (Plaut, 1951) or mitochondria (Fischer, 1955) from biotin deficient rats, the incorporation of $C^{14}O_2$ into acetoacetate is reduced in the first case, and the evolution *in vitro* of isovalerate to acetoacetate is blocked in the second case

The site of biotin involvement is precisely at the stage of the conversion of β hydroxyisovaleryl CoA to acetoacetate, a step involving the following operations (Woesmer *et al*, 1958)

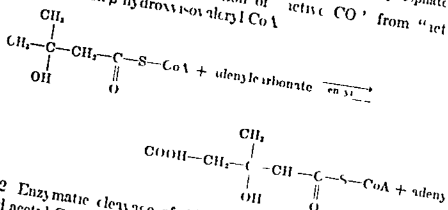
1 Enzymatic carboxylation of β hydroxyisovaleryl CoA to β hydroxy-

β methylglutaryl CoA This reaction is divided into two pha[ses] catalyzed by an enzyme

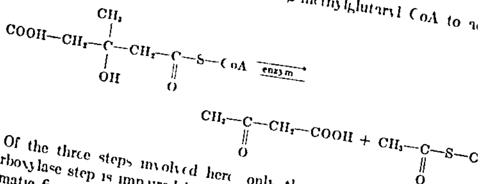
Phase a Activation of CO_2 reacting with ATP to give, [the] active adenyl carbonate"



Phase b Transfer and fixation of "active CO" from "act[ive adenyl car]bonate" on β hydroxyisovaleryl CoA



2 Enzymatic cleavage of β hydroxy β methylglutaryl CoA to a[ceto...] and acetyl CoA



Of the three steps involved here only the β hydroxyisovaleryl CoA carboxylase step is impaired by biotin deficiency that is to say, the enzymatic fixation of the CO_2 on the β hydroxyisovaleryl CoA disappears completely from the liver extracts of biotin deficient rats (Woesmer *et al*, 1958) It is not clear how biotin is linked with this carboxylase, if at all Indeed all the attempts to restore *in vitro* the activity of β hydroxyisovaleryl CoA carboxylase by the addition of biotin biocytin, N carbamyl glutamate, etc to liver extracts of biotin deficient rats have failed (Woesmer *et al*, 1958)

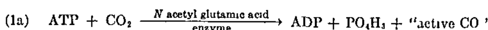
iii Aspartic acid Although biotin deficient microorganisms cannot deaminate aspartic acid (see Lichstein 1951), rats deprived of biotin can deaminate this amino acid very well The normal course of the catabolism of aspartic acid is in no way disturbed and a heavy overload of aspartic acid is easily catabolized

The excretion of urinary amino acid nitro[gen]

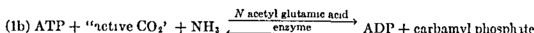
tion of the degree of catabolism of amino acids, is not increased and is the same as that observed after an overload of alanine given as an amino acid control (T Terroine and Rombauts, 1953)

iv Ornithine and citrulline The power to synthesize citrulline from ornithine *is reduced by 50 % in* liver homogenate from biotin deficient rats This was the initial observation by McLeod and Lardy (1949) showing the participation of biotin in this synthetic process Citrulline synthesis is a complex operation comprising several successive phases which can be shown schematically in the following manner (Grisolia and Cohen, 1952, Marshall *et al* , 1956, Jones *et al* , 1955, Burnett and Cohen, 1957, Metzenberg *et al* , 1958)

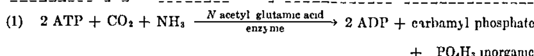
(1) Synthesis of carbamyl phosphate comprising the following two successive stages



$$\text{(1a)}\quad ATP + CO_2 \xrightarrow[\text{enzyme}]{N\text{ acetyl glutamic acid}} ADP + PO_4H_3 + \text{"active CO"}$$

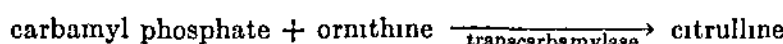


$$\text{(1b)}\quad ATP + \text{"active } CO_2\text{"} + NH_3 \underset{\text{enzyme}}{\overset{N\text{ acetyl glutamic acid}}{\rightleftarrows}} ADP + \text{carbamyl phosphate}$$



$$\text{(1)}\quad 2\,ATP + CO_2 + NH_3 \xrightarrow[\text{enzyme}]{N\text{ acetyl glutamic acid}} 2\,ADP + \text{carbamyl phosphate} + PO_4H_3 \text{ inorganic}$$

(2) Reaction of transcarbamylation



$$\text{carbamyl phosphate} + \text{ornithine} \xrightarrow[\text{transcarbamylase}]{} \text{citrulline}$$

There is still no definite agreement on the exact nature of the carbamyl group donor, whether it is the carbamyl phosphate *sensu stricto* (Jones *et al* , 1955) or a slightly different derivative (Grisolia *et al* , 1955) It is not yet known, moreover, whether the two phases (a and b) of reaction (1) are governed by a single enzyme or by two distinct enzymes (Metzenberg *et al* , 1958)

Taken as a whole the above mentioned reactions correspond to reactions which take place in the liver of mammals, and also of the frog

In *microorganisms reaction* (1) *in particular* takes place in a different way



$$ATP + NH_3 + CO_2 \rightleftarrows ADP + \text{carbamyl phosphate}$$

The two most striking differences between the two types of citrulline synthesis in microorganisms and in mammals are concerned with the following points (a) complete reversibility of carbamyl phosphate synthesis and transcarbamylation in microorganisms, and irreversibility in higher animals Here, although reaction (1b) is reversible, reaction (1a) is irreversible, and as a result reaction (1) becomes irreversible (Jones *et al* , 1955), (b) necessity for the presence of a cofactor for the synthesis of

carbamyl phosphate in higher animals This cofactor is either acetyl glutamate as a natural cofactor, or carbamyl glutamate as a synthetic cofactor (Hall *et al*, 1958) Microorganisms, on the other hand, do not need such cofactors (Marshall *et al*, 1955)

Biotin is very probably implicated in the two stages of citrulline synthesis carbamyl phosphate synthesis and transcarbamylation

Biotin deficient rat liver homogenate can synthesize citrulline from ornithine normally if carbamyl glutamate, a cofactor of carbamyl phosphate synthesis in mammals, is present, as mentioned above (Feldott and Lardy, 1951) Thus, in mammals, biotin seems to be involved in carbamyl phosphate synthesis by a mechanism which is still to be discovered

A cell free extract of biotin-deficient *S lactis* 8043 can only weakly synthesize citrulline from ornithine and carbamyl phosphate (Estes *et al*, 1956) Biotin deficiency in this microorganism would slow down the second step in citrulline synthesis—that of transcarbamylation In no case, however, does the *in vitro* addition of free or bound biotin to biotin deficient rat liver homogenate or to a cell free extract of *S lactis* 8043 deprived of biotin restore the normal rate of synthesis of citrulline from ornithine This rate is re-established very quickly when biotin is reintroduced into the culture medium of *S lactis* or in the diet of deficient rats (Feldott and Lardy, 1951, Estes *et al*, 1956) This restoration of the transcarbamylation reaction in the biotin-deficient cells of *S lactis* occurs after a short (2 hour) incubation of these cells with phosphate buffer, glucose, biotin, and numerous amino acids This latter effect would suggest that the restoration is accompanied by the synthesis of the whole of the enzyme, rather than only a fraction of it (Sund *et al*, 1958)

Moreover, it has been shown that highly purified preparations of the ornithine citrulline enzyme of *S lactis* 8039 contains only 1 part of biotin in 5,000 000 parts of purified enzyme This evidence also suggests that biotin exerts its action during the synthesis of the enzyme, and is not a component of the enzyme (Ravel *et al*, 1959) In fact, the role of biotin in citrulline synthesis remains to be defined precisely Does biotin participate effectively in the two phases of the synthesis reaction as the above experiments would suggest? Does it on the other hand participate directly in these reactions as an enzymatic cofactor, or only indirectly by assisting in the synthesis of the enzymatic systems which are involved?

The conversion of ornithine to citrulline is of great interest since it constitutes the first stage in urea formation (Krebs and Henseleit, 1932) It was thus of particular interest to find out whether biotin deficiency would cause a decrease in the urea formation rate The results of T Terroine and Rombauts (1952) show that biotin deficiency does indeed slow the formation of urea in the rat But this slowing is quite feeble, only about 8 %, and

cannot compare with the 50% reduction observed *in vitro* in the conversion of ornithine to citrulline It is reasonable to think that, in spite of an 80% reduction in the hepatic concentration of biotin due to the deficiency (T Terroine, 1956b), the deficient organism has nevertheless a sufficient supply of biotin and enzyme to permit almost normal formation of urea *in vivo*

This lack of agreement between the results of *in vivo* and *in vitro* experiments is not only quantitative, but also a disagreement of principle T Terroine and Rombauts (1952) showed that the slight reduction in the formation of urea observed in the living rat is not a primary disorder of biotin deficiency, but only a secondary reflection of an initial acidosis It has been mentioned that biotin deficiency produces a strong hyperpyruvic emia *in vivo* This creates a state of acidosis which the organism combats by a very strong overproduction of ammonia, resulting in a reduction of urea formation (T Terroine and Rombauts, 1953)

Thus, the *in vitro* studies indicate that biotin is perhaps an indirect but very important agent in controlling one of the stages of urea formation On the other hand, the *in vivo* studies show that biotin plays only a secondary role in the control of urea formation The validity of the two groups of results being incontestable, their divergence only demonstrates again the frequent impossibility of integrating the methods of research *in vitro* and *in vivo* The former isolates a process, the latter registers the resultant of multiple and often contradictory effects The former shows the theoretical role of biotin in one of the stages of urea formation, the latter shows the actual part biotin plays in this process Far from standing in opposition to each other, the two groups of researches are on the contrary complementary

v Serine The earlier studies have shown that the deamination of serine is impaired in some microorganisms by biotin deficiency (see Lichstein, 1951) Deamination of this amino acid is also reduced *in vitro* in liver homogenates from biotin deficient rats Moreover, in such homogenates, the power to synthesize serine by fixation of CO_2 on ethanolamine is also decreased (Nadkarni and Sreenivasan, 1957) The latter reaction is particularly interesting since it constitutes a new example of the intervention of biotin in CO_2 fixation Here again, serine synthesis by liver homogenates from deficient rats is not restored by the addition of biotin *in vitro*, but only after the injection of the vitamin into the living deficient animal (Nadkarni and Sreenivasan, 1957) It is interesting to note that folic acid deficiency has no effect on the deamination of serine, but reduces the decarboxylation of this amino acid by rat liver The latter reaction is not modified by biotin deficiency (Nadkarni and Sreenivasan, 1957)

vi Methionine Biotin deficiency reduces considerably the incorporation of methionine S^{35} into the proteins of the blood, liver, brain, muscle, and skin of the rat The administration of 10 μg of biotin per day on two suc

cessive days prior to the injection of methionine S^{35} restores to normal the incorporation of methionine in these tissues (Kritzman *et al*, 1953)

vii Tryptophan At present there is disagreement as to the role of biotin in the catabolism of tryptophan, different stages of which are shown in Fig 2

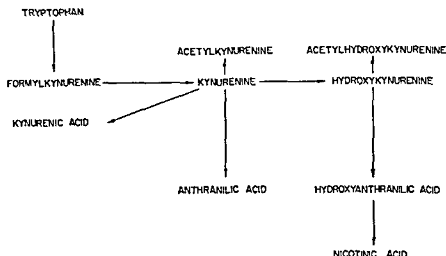


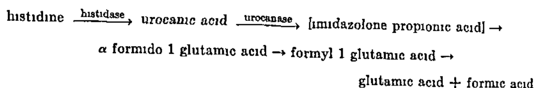
Fig 2 Outline of the pathway for conversion of tryptophan into nicotinic acid according to Dalgliesh (1955)

On the one hand, biotin seems to be actively involved in this metabolism, for in biotin deficiency the first stage of the degradation of tryptophan (the conversion of the imino acid into formylkynurenine) appears to be blocked in a mutant, *Neurospora crassa* 39401 (Sundaram *et al*, 1954) Also, the biotin-deficient rat is incapable of converting in appreciable quantity an excess of tryptophan added to its diet as niacin derivatives (Sundaram and Sarma 1955) On the other hand, Dalgliesh (1955) finds that biotin deficient rats, after having received an overload of tryptophan, excrete as much kynurenine, hydroxykynurenine and anthranilic acid as the controls These irreconcilable results indicate that further research is needed

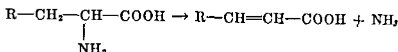
In another connection, Poznanskaja (1958) found that 1 hour after the intraperitoneal introduction of *dl* tryptophan, the tryptophan peroxidase activity in the livers of biotin deficient and control rats both increased to the same extent The tryptophan peroxidase activity of liver slices incubated with tryptophan for 1 hour was only slightly less in the deficient rats Thus, biotin deficiency does not affect the adaptative formation of tryptophan peroxidase in the rat

viii Histidine One of the principal processes of histidine degradation

in mammals—and perhaps the most important (Abrams and Borsook, 1952, Mehler and Tabor, 1953)—involves a direct enzymatic deamination in urocanic acid with formation of glutamic acid, as is shown by the following reactions



The first stage of degradation of histidine to urocanic acid has the same pattern as the microbial catabolism of aspartic acid In both cases, there occurs a reaction of the type



R being either the carboxyl of aspartic acid or the imidazole ring of histidine

However, if biotin deficiency reduces or inhibits the bacterial deamination of aspartic acid (see Lichstein, 1951), it does not attenuate the liver histidase activity in the rat, on the contrary, it is increased as in pyridoxine deficiency Biotin deficiency does not modify, on the other hand, the urocanase, glutamico aspartico transaminase or the rhodanese activity (Baldridge and Tourtelotte, 1957) In comparison, it is interesting to note that folic acid deficiency considerably decreases urocanase activity, but not liver histidase activity in the rat (Baldridge, 1958)

The stimulation of the latter enzyme, as a consequence of biotin deficiency, can be compared with that of L-amino acid oxidase of *Neurospora crassa* placed in a low-biotin medium (Thayer and Horowitz, 1951) In both cases biotin does not seem to participate specifically in histidine metabolism, but is probably indirectly involved in the protein synthesis of histidase or of L amino acid oxidase (Baldridge and Tourtelotte, 1957, Thayer and Horowitz, 1951)

b Metabolism of Creatine Bodies As opposed to the work concerning metabolism of individual amino acids quoted above, the studies on the metabolism of creatine bodies were carried out in the rat only

i Muscle creatine Is the "kangaroo" gait so characteristic of the biotin deficient rat connected with a disturbance in muscle creatine metabolism? All authors agree that there is a relationship between the atrophy and the histological changes of the muscle observed in biotin deficiency (Sullivan *et al*, 1942, Shaw and Phillips, 1942, Pizzolato and Beard, 1945), but they are not in agreement as to the rate of formation of muscular creatine in the deficient rat Some find it less (Pizzolato and Beard, 1945)

and others find it greater (Nielsen and Elvehjem, 1942, Lazere *et al*, 1943) than the normal rate It should be added too, that biotin does not seem to participate in the synthesis *in vitro* of creatine by a liver homogenate (Cohen, 1953)

ii Urinary creatinine Since the level of creatininuria is generally considered to be reflection of muscular creatine degradation, its determination in biotin deficiency may produce indirect information on muscular creatine metabolism in this vitamin deficiency But here again, the results do not agree Ricceri (1958a) observed a slow decrease in the urinary creatinine excretion during biotin deficiency However, the author did not give the values of creatinine excreted in relation to the weight of the animal, as would seem logical considering the close relationship previously mentioned between urinary creatinine and muscular activity Taking this into account T Terroine and Rombauts (1952) found, on the contrary, a considerable increase of the average daily excretion of creatinine per 100 gm of body weight in endogenous or exogenous metabolism If confirmed, this increase of creatininuria would be in accord with the increase in muscle creatine in biotin deficiency observed by certain authors, since an increased degradation of muscular creatine would correspond to an increase of urinary excretion of creatinine

iii Urinary creatine The excretion of urinary creatine in biotin deficiency is increased considerably (T Terroine and Rombauts 1952), but creatinuria is nonspecific and is caused by many interventions (E F Terroine, 1938)

Thus in summary, it may be said that the effect of biotin on the metabolism of creatine bodies is not clear, nor is it unanimously established

c General Processes of Protein Metabolism Information on this subject is extremely scarce It deals, on the one hand, with the possible implication of biotin in the transamination processes, and on the other with its possible participation in protein synthesis

i Transamination The activity of aspartico-α ketoglutarico transaminase drops by 32%, and that of glutamico pyruvico transaminase is reduced by 7% in the liver of biotin deficient rats, but only the reduction of the activity of the first enzyme is significant (Rossi *et al*, 1957)

ii Protein synthesis (1) Enzymes To explain the role of biotin in enzymatic reactions it has been suggested that this vitamin might participate in the synthesis of the protein portion of the enzymes, for instance in microorganisms, in the following enzymatic activities transcarbamylase, involved in the conversion of ornithine to citrulline (Sund *et al* 1958) succinyl CoA decarboxylase, participating in the conversion of succinic acid to propionic acid (Chambers and Delwiche, 1954), histidase and L amino acid oxidase (Baldridge and Tourtelotte, 1957, Thayer and Horowitz,

1951), lactic dehydrogenase, serine oxidase, fatty acid dehydrogenase (Gothoskar and Sreenivasan, 1953). But in each instance, the notion that biotin plays a part in the synthesis of apoenzymes is either hypothetical or is based on insufficient experimental data.

The only thorough research on the possible role of biotin in the synthesis of the protein fraction of an enzyme is that of Poznanskaja (1957). This author observed that biotin deficiency depresses considerably the *in vitro* formation of amylase by chick pancreas slices. The intraperitoneal injection of 100 μg of biotin 24 hours prior to killing was enough to restore the normal synthetic activity of this enzyme.

The synthesis of proteins such as amylase is an endothermal process which is coupled with the cleavage of high energy bonds of ATP. This process is impaired under conditions where cell respiration and oxidative phosphorylation are depressed. With this fact in mind, Poznanskaja (1957) studied the effects on amylase synthesis of supplementing chick pancreas slices with some of the tricarboxylic acid cycle intermediates. The addition of pyruvic acid had no effect, as was to be expected since biotin deficiency inhibits the transformation of pyruvate into oxalacetate and its subsequent oxidation through the tricarboxylic cycle. On the other hand, γ ketoglutarate restored amylase synthesis by pancreas slices from biotin-deficient chicks to almost the normal rate. Supplementation with aspartate resulted in about half the synthesis observed in the case of α ketoglutarate. The author thought that these results further supported the view that biotin is not only essential in the synthesis of 4 carbon dicarboxylic acids in the respiratory cycle, but is also concerned with their further transformation into α ketoglutarate. From these results Posnanskaja (1957) deduced that biotin does not participate directly in protein synthesis, but acts, perhaps as a coenzyme, in the synthesis of dicarboxylic acids of the citric acid cycle. These acids are essential for the processes of oxidative phosphorylation which, in turn, provide the energy requirements for protein synthesis. Thus, biotin seems to participate indirectly in protein synthesis by contributing energy necessary for this synthesis.

(2) *Serum albumin.* Liver slices from biotin deficient chicks synthesize serum albumin very weakly. This synthesis is restored to normal either *in vitro* by addition of glutamine (but not α ketoglutarate) or *in vivo* by administration to the deficient animal of 100 μg of biotin 24 hours prior to sacrifice (Posnanskaja, 1957). As in the case of amylase, Posnanskaja (1957) thought that biotin is indirectly involved in the synthesis of serum albumin. It participates in the synthesis of dicarboxylic acids and therefore supplies the energy needed for the synthesis of serum albumin.

(3) *Antibodies.* Only a few studies have been made on the consequences of biotin deficiency upon the formation of antibodies. After either diphtheria

toxoid, or human erythrocytes were injected as antigens into the biotin deficient rat, the antibody formation was strongly inhibited in the first study (Pruzansky and Axelrod, 1954) and only slightly in the second study (Carter and Axelrod, 1948). On the other hand, biotin deficiency in no way decreased the production of antibodies in chicks after the administration of urease (McCoy and Nair, 1954) or pig gamma globulin (McCoy and Sensenich, 1945). The inhibitory action of pyridoxine or pantothenic acid deficiency is comparatively much greater. The intimate mechanism by which biotin deficiency, and other vitamin deficiencies, inhibit the production of certain antibodies is still unknown. Axelrod and Pruzansky (1955) have advanced several hypotheses to explain this.

5 Nucleic Metabolism

a Purine Synthesis. Biotin participation in the synthesis of purine bases seems evident in microorganisms but is debatable in mammals.

i Metabolism of microorganisms. A culture of *Saccharomyces cerevisiae* (mutant Fleischman strain 139) deprived of biotin, accumulated an aromatic amine identified as 4 aminoimidazole. By the addition of biotin to the culture medium the 4 aminoimidazole gave rise to a new substance, 4 amino 5 imidazole carboxamide, which represented a supplementary stage of purine synthesis. As shown in Fig 3, this constituent is formed by the incorporation of CO_2 and of the NH_2 radical into the 4 aminoimidazole; the reaction thus giving rise to carbon 6 of the future purine ring has not yet been definitively established.

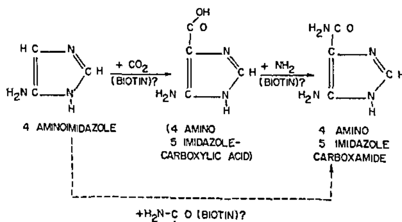


FIG 3 Potential sites of biotin action in the conversion of 4 aminoimidazole to 4 amino 5 imidazole carboxamide by *Saccharomyces cerevisiae*. From Moat *et al* (1956)

Whether biotin contributes only to the incorporation of CO_2 or also to that of the radical NH_2 has not yet been determined (Moat *et al* 1956).

The use of the 4 aminoimidazole for purine synthesis has also been blocked with another biotin deficient strain of *S cerevisiae*, the mutant 855 (Lindegren and Lindegren, 1947) Finally, Chamberlain *et al* (1952) have observed in a low biotin yeast medium, the accumulation of an arylamine, chemically unidentified but probably identical with the 4 aminoimidazole

Moreover, the synthesis of riboflavin by *Aspergillus oryzae* is considerably attenuated by biotin deficiency The addition of biotin to the medium restores this synthesis to its normal rate Here again biotin is probably implicated in the incorporation of CO_2 in the synthesis of the purine bases which are known precursors of riboflavin (Tirunarayanan *et al*, 1954)

ii Animal metabolism In mammals, the indirect evidence indicates that biotin is involved in purine synthesis Biotin deficient rats which received an injection of $NaHC^{14}O_3$ fixed less $C^{14}O_2$ in the guanine and adenine of their viscera than did the control animals (MacLeod and Lardy, 1949)

It is important to emphasize that when biotin is implicated in purine metabolism, it functions through its dominant biochemical property That is to say, it contributes to the incorporation of CO into a given molecule

The *in vivo* studies are not in agreement, on the other hand, that biotin participates in the synthesis of purine bodies Were it so in the biotin deficient rat, for instance, then the urinary elimination of the purine bodies, and allantoin in particular, would be decreased

This was observed by Ricceri (1958a) The administration of biotin raised the allantoin and uric acid excretion rate in deficient rats The results of T Terroine (1956c) are more finely drawn In fact, under experimental conditions similar to those of Ricceri, the average daily excretion of *N* allantoin also dropped considerably in biotin deficiency However, when it was calculated per 100 g body weight, as it is rational to do, the daily elimination rate of allantoin in the urine was identical in the deficient and in the control groups The uric acid excretion was also identical The purine oxidation coefficient is consequently normal The exogenous nucleic metabolism also remained quite normal following a large addition of sodium ribonucleate in the diet The ability to synthesize the purine bodies excreta in the living rat thus does not appear to be affected by biotin deficiency (T Terroine, 1956c) In the rat, the effects of biotin deficiency on the activity of the principal enzymes involved in the metabolism of purine bodies shed no light on the possible relationships between biotin and purine catabolism Ricceri (1958c) reported that the activity of liver xanthine oxidase is increased by biotin deficiency, but T Terroine (1956c) observed no significant change The activity of liver uricase of the deficient animal was strongly stimulated, suggesting that biotin regulates *in vivo*, directly or indirectly, either the synthesis or destruction of uricase

(Ricceri, 1958b) The stimulation of the activity of xanthine oxidase and uricase, observed in biotin deficiency *in vitro* by Ricceri, does not agree with the above results obtained *in vivo* in which biotin deficiency decreased or did not change the elimination rate of the products of the catabolism of purine bodies It should be added that the activity of adenosine and guanosine liver deaminase is not modified in the absence of biotin (Ricceri, 1958b)

b Nucleic acid synthesis Here also the results are contradictory

i Metabolism of microorganisms It seems that biotin decreases the synthesis of nucleic acids in microorganisms On the one hand, an excess of biotin in the medium of *Lactobacillus casei*, *L arabinosus*, or *B subtilis* considerably reduced the synthesis of ribo and deoxyribonucleic acids by these organisms, perhaps provoking a disorder in the processes of nucleotide synthesis (Gothoskar *et al*, 1954) Inversely, a biotin deficient medium seemed to increase slightly the content of ribo and deoxyribonucleic acids with *L casei* (Prusoff *et al* 1948)

ii Animal metabolism A decrease was noted in the ribonucleic acid content of the cytoplasm of the liver, heart, and kidneys of newborn rats from biotin deficient mothers, while the deoxyribonucleic acid content remained unchanged (Cooper and Brown, 1958)

6 *Potassium Metabolism*

The relationship between biotin and potassium metabolism seems to be species specific

When young calves are maintained on a potassium deficient diet, they show a progressive paralysis, which begins in their hind legs and spreads to their fore legs neck, and finally, respiratory system (Thipse *et al*, 1948) An identical paralysis is seen in the dog when deprived of potassium (Gover Smith *et al*, 1950)

The paralysis can be cured in these two species by the administration of an excess of potassium or of biotin (Thipse *et al*, 1948, Gover Smith *et al*, 1950) Biotin is, however, less effective than potassium in the paralyzed dog (Gover Smith *et al*, 1950) It is interesting to note moreover, that while biotin cures the paralysis of the potassium deficient calf, it has no effect in this species upon muscular dystrophy caused by a large addition of cod liver oil to a vitamin E deficient diet (Blaxter *et al*, 1953)

These results taken as a whole suggest that there may be an interdependence between biotin and potassium in the dog and the calf To confirm this, it will be necessary to study the consequences of the administration of an excess of potassium to dogs or calves deprived of biotin and to define the intimate mechanism of this biotin potassium interrelationship

The paralysis caused by deprivation of potassium, which is cured in the

calf and the dog by the administration of biotin, is of the ascending type It differs from the hypertonic type of paralysis characteristic of biotin deficiency, particularly that seen in the rat and the pig (Cunha *et al*, 1946) Could it not be suggested, nevertheless, in the light of the above results, that the abnormal gait of the biotin deficient animals may be due to a potassium depletion of the deficient organism? It is not so in the biotin deficient rat, in which, however, the potassium content of the muscle falls systematically Though constant, this decrease is not very important since it is only about 15% There is no parallelism between the severity of the paralysis and the potassium depletion of the muscle The abnormal gait of the biotin deficient rat thus appears to be independent of potassium metabolism (Servigne and T Terroine, 1954) Moreover, the rat seems to have a specific behavior with regard to this metabolism, for potassium deficiency never produces paralysis in the rat (Gover Smith *et al*, 1950) and the symptoms in the potassium deficient rat are in no way relieved by an excess of biotin (Kornberg and Endicott, 1946)

III Biotin and Endocrine Glands

1 *Thyroid and Adrenals*

On the basis of histological examination it would appear that the activity of the thyroid and adrenal glands is not affected even with severe biotin deficiency in the rat (Shaw and Phillips, 1942, Katsh *et al*, 1955, Delost and T Terroine, 1956)

2 *Genital Tract and Reproduction*

The following studies will show that biotin deficiency provokes disorders in all phases of reproduction in the rat and in the bird

a Morphology and Function of the Genital Glands, Gametogenosis i *The male rat* The serious changes in the male genital tract produced by biotin deficiency can be summarized briefly (1) decrease in size of the testis (Shaw and Phillips, 1942, Manning, 1950, Okey *et al*, 1950, Delost and T Terroine, 1956, Communal, 1957) due to biotin deficiency *sensu stricto* and not to a simple decrease in appetite, for it does not appear in the pair-fed controls (Delost and T Terroine, 1956), (2) cryptorchidism (Manning 1950, Bishop and Kosarick, 1951) linked (Bishop and Kosarick, 1951) to a general state of malnutrition produced by biotin deficiency but not specifically due to it, (3) lesions in the seminiferous tubules (Shaw and Phillips, 1942, Delost and T Terroine, 1956, Communal, 1957), (4) serious and rapidly developing disorder in the spermatocytes, which degenerate (Shaw and Phillips, 1942, Katsch *et al*, 1955, Delost and T Terroine, 1956, Tharanne, 1956, Communal, 1957), (5) reduction of the number of

spermatozoids, which may sometimes be degenerate (Okey *et al*, 1950, Delost and T Terroine, 1956)

The disorder of the genital tract seems to be due primarily to a decrease in activity of the interstitial cells rather than to a direct effect on the target organs Indeed, in deficient rats, the interstitial cells appear histologically to be hypoactive and the general changes of the accessory glands and the spermatic ducts suggest a deficiency in male hormone (Delost and T Terroine, 1956)

Respecting the seminiferous tubules and the germinal cells, it is difficult at present to define the intimate mechanism leading to the lesions Biotin deficiency might act directly on the nutrition of the cells of the seminiferous tubules and spermatozoids or might be involved also in causing hypofunction of the hypophysis (Delost and T Terroine, 1956)

Disorders of the genital tract appear early, in the third or fifth week of vitamin deficiency, at a time when the symptoms of vitamin deficiency are not apparent or are very slight Moreover even in acute biotin deficiency, complete atrophy of the testis or the genital tract is never seen (Delost and T Terroine, 1956)

ii Case of the female rat Frequently the uterus of the adult rat is hyperemic and distended with colorless fluid Microscopically however, the uterine glands are reduced in number and the epithelium shows little proliferation (Okey *et al*, 1950)

There are usually atretic changes in the ovaries of rats with apparently normal hair The ova in the larger follicles seem to have undergone degenerative changes The degree of atresia is greatest in the animals which have been on the biotin deficient diet for the longest time (Okey *et al*, 1950)

b Embryonic Development i Embryonic malformations The most characteristic effect of a vitamin deficiency on embryonic development is of course in the production of malformations, since these correspond to disturbances of the specific morphogenetic mechanisms of this period But the effects of biotin deficiency on embryonic development are totally different in the rat as compared to the chick

(1) Chick embryo The malformations, which may appear as soon as the eighth day of incubation, are essentially the following deformation of the beak into a "parrot beak," syndactilism malformation of the bone causing a general shortening, and finally, a perosis which gives an abnormal position of the feet, these becoming stiff and stretched out behind so that at the birth the animal cannot stand up (Cravens *et al* 1944, Couch *et al*, 1948 1949)

(2) Rat embryo In the biotin deficient rat 90% of the implanted eggs give normal fetuses, though the maternal deficiency is so acute that certain

observed no stimulatory action of biotin on the *in vitro* growth of the nerve cells of chicken embryo

Besides, Pellegrino (1957) observed that biotin treatment inhibited a predisposition of the dog to experimental reflex epilepsy, whereas biotin sulfone, an antagonist of the vitamin, favors this predisposition The author suggested that the predisposition to experimental reflex epilepsy is connected with a disorder in the decarboxylation of oxalacetic acid, causing an accumulation of the latter in nerve tissues This disorder would not take place in the presence of biotin participating in the decarboxylation of this acid This seems to be the first attempt to establish a link between certain nervous lesions and a biochemical disorder in the absence of biotin

From an anatomical and histological study of symptoms of rats deficient in biotin, Rose *et al* (1956) concluded that the numerous lesions observed were probably due to the parasympathetic cholinergic system, and secondarily to the sympathetic system In the sympathetic system Tharanne (1956) observed serious lesions during the course of alopecia caused by biotin deficiency

V Biotin and the Cutaneous System

The skin is an excellent indicator of the state of health of the organism Biotin deficiency, more than other vitamin deficiencies, is very harmful to the integrity of the skin and hair

1 *Skin*

Biotin deficiency gives rise to dermatitis in many animal species mouse, rat, pig, poultry, man This dermatitis has different characteristics in these different species Thus, for instance, hyperkeratosis is very pronounced in the rat, but moderate in mouse A detailed account of the histological analyses of the skin of the biotin deficient rat and mouse will be found in the work of Gyorgy (1954)

The similarity between the cutaneous manifestations of the biotin deficient rat and the syndrome of seborrheic dermatitis in human cases, and in particular Leiner's disease in young infants has been pointed out by several authors (Gyorgy, 1941, Brown, 1948, Swejcar and Homolka, 1950, Tripputi, 1950, Berger, 1950) Leiner's disease seems to be associated with a deficiency in biotin due both to the low content of this vitamin in human milk and to a loss of biotin by the infant because of persistent diarrhea, which is part of the clinical picture of this disease It is known that cures of seborrheic dermatitis of infants have been obtained by the administration of biotin (Petrocini and Debernardi, 1954, Kokil 1954, Nisenson, 1957, Gautier *et al*, 1957)

On the other hand, the results following the administration of biotin to

adults with seborrheic dermatitis are contradictory sometimes nil (Oppel, 1948), sometimes favorable (Colli, 1958) It is possible that the etiologies of seborrheic dermatitis in the adult and in the infant are different

2 *Hair*

a Alopecia Acute biotin deficiency in the rat and the mouse causes alopecia which is often generalized over the body The hair seems to grow at a normal rate (Rauch, 1952, Montagna, 1956), but it breaks at the base because keratin formation is faulty (Rauch, 1952, Montagna, 1956, Tharrane, 1956) X ray analysis of the hair from a biotin deficient rat reveals no abnormality of structure (T Terroine and Legrand, unpublished data) Alopecia in biotin deficiency seems to be linked with sex, according to Okey *et al* (1950) Indeed, it seems to be more serious and to appear more rapidly in the male rat than in the female In a very interesting histological study Tharrane (1956) showed that the sympathetic is involved in the alopecia of the biotin deficient rat this involvement becomes more serious as the alopecia becomes acute Thus this work raises the question of sympathetic lesions in alopecias

b Depigmentation In biotin deficiency, the black hair of the rat or mouse becomes brown or even gray (Gyorgy, 1941, Sullivan and Nicholls 1942, Emerson and Keresztesy 1942, Wilson *et al*, 1949, Rauch 1952, Quevedo, 1956) Quevedo (1956) observed that melanocytes persist in the hair follicles of the biotin deficient mouse and that their histological structure is comparable with that of the colorless cells of the white mouse The depigmented melanocytes retain the ability to respond to the dihydroxy phenylalanine oxidase test The exact role of biotin in the process of depigmentation is not known It is interesting to recall that pantothenic acid also plays a role in this process Perhaps this is an exterior sign of an interrelationship between the two vitamins

VI Vitamin Interrelationships of Biotin

The only vitamin interrelationships of biotin that have been demonstrated are those which link this vitamin with ascorbic acid The other vitamin relationships of biotin which have been suggested are far from established and will be mentioned here only briefly

1 *Biotin Pyridoxine Relationship*

MacKay and Barnes (1941) observed that the simultaneous administration of a large amount of C_{18} unsaturated fatty acids and pyridoxine to biotin deficient rats in an egg white diet, improved remarkably the general state and growth and reduced the severity of the symptoms However, Johnson *et al* (1952) could not confirm the favorable action of pyri

doxine in biotin deficiency under similar conditions Nielsen and Elvehjem (1942) observed no protective action of pyridoxine against the abnormalities of gait in the biotin deficient rat Thus, a biotin pyridoxine relationship has not been confirmed

2 Biotin Riboflavin Relationship

A large excess of riboflavin was given either orally or by intraperitoneal injection without affecting the paralysis of the biotin deficient rat (Nielsen and Elvehjem, 1942) On the other hand, optimum synthesis of biotin in the intestinal tract of the rat requires the presence of riboflavin (Nielsen and Elvehjem, 1942) Bhagvat *et al* (1949) observed *in biotin deficient* rats an important and significant increase in the riboflavin content of the liver The links between biotin and riboflavin, brought to light by the two preceding observations, need to be more thoroughly studied

3 Biotin Thiamine Relationship

Here again the experimental facts are few and should be increased The addition of extra thiamine to the medium of *Phycomyces blakesleeanus* enhances the power of this organism to synthesize biotin (Schopfer, 1943) On the other hand, Bhagvat *et al* (1949) observed an important drop in the thiamine concentration of the liver of the biotin deficient rat

4 Biotin Pantothenic Acid Relationship

Biotin added to a pantothenic acid deficient diet had some protective effect against this deficiency in the rat (Emerson and Wurtz, 1944) and in the pig (Colby *et al*, 1948) In the first case the biotin supplement attenuated the severity of the deficiency symptoms whereas in the second, it prolonged life, but hastened the development of the deficiency symptoms Pantothenic acid utilization by the rat seems to depend on the simultaneous presence of biotin and folic acid (Wright and Welch, 1943)

5 Biotin Folic Acid Relationship

Luckey *et al* (1955) showed with germ free rats that biotin participates in the biosynthesis of folic acid However, Halevy and Guggenheim (1958), working with non germ free animals were unable to confirm these results They stated that the addition of biotin increases neither the folic acid nor the citrovorum factor content, which are lower in the rat treated with succinylsulfathiazole It did not improve the conversion of pteroylglutamic acid into the citrovorum factor

6 Biotin Ascorbic Acid Relationship

Ascorbic acid administered to young rats as 0 1 % of the diet throughout the deficiency in biotin decreases or abolishes all the specific clinical symp

toms of this deficiency in a truly spectacular manner (T Terroine, 1954) Thus, before the 92nd day of this deficiency, all the untreated animals deprived of biotin had a more or less widespread alopecia, 90% of them had "spectacle eyes," and 50% had the "kangaroo" gait However, 70% of the deficient animals which received ascorbic acid had no alopecia, and none had "spectacle eyes" nor the "kangaroo" gait

Furthermore, the survival time is remarkably prolonged by giving ascorbic acid to deficient animals On the 92nd day of deficiency, all the animals not receiving ascorbic acid were dead but 70% of those receiving it were still alive Only after the 125th day of the treatment was the mortality in this group high (60%) It is also worth mentioning that half the biotin deficient rats treated with ascorbic acid showed no apparent symptom of deficiency at the time of death

The body weight and appetite were little improved, on an average, by treatment of the deficient group by ascorbic acid (T Terroine, 1954)

Finally, in the metabolic field, ascorbic acid attenuated somewhat the excessive urinary excretion of ammoniacal nitrogen characteristic of biotin deficiency (T Terroine, 1954)

On the whole, although the protection offered by ascorbic acid against biotin deficiency was incomplete and temporary, it is quite remarkable

The protective role of ascorbic acid is also seen in microorganisms The addition of ascorbic acid to the culture medium of biotin subdeficient *L arabinosus* always improved the growth of this microorganism (T Terroine 1958) In the rat as in *L arabinosus*, the supplementary power of ascorbic acid toward biotin was exercised only at pharmacodynamic doses

The mechanism of the vicarious activity of ascorbic acid in biotin deficiency is still unknown When biotin deficiency was continued in the rat up to the moribund stage the total ascorbic acid in the liver and adrenals decreased only 17% (T Terroine, 1954) Thus, it is not very likely that the making up of this moderate deficit in "vitamin C" can explain the protective action of ascorbic acid

The suggestion of a functional substitution of ascorbic acid for the missing biotin seems tempting *a priori*, in the light of the above mentioned hypothesis of Lichstein (1951), according to which biotin would exercise a unique role of hydrogen carrier in the enzymatic reactions in which it participates However, it is first necessary to know whether ascorbic acid functions as a hydrogen carrier in animal metabolism

The powerful protection exerted by ascorbic acid against biotin deficiency is not an exclusive specific power of this factor It is shared, to various degrees, with other redox agents Thus, an isomer of ascorbic acid, isoascorbic acid, practically devoid of any antiscurvy activity (Demole, 1934), stimulates the growth of biotin subdeficient *L arabinosus* equally as well as ascorbic acid (T Terroine, 1958) Also the administration,

throughout the biotin deficiency, of methylene blue or neutral red to the rat considerably protects him against the effects of this deficiency This protection is much less effective, however, than that offered by ascorbic acid (T Terroine, 1954) These observations suggest that ascorbic acid must exercise its substitute activity in biotin deficiency not as a vitamin but as a nonspecific pharmacodynamic redox agent

The protective ability of ascorbic acid is of special interest when it is remembered that it exercises its effects against numerous other vitamin deficiencies (T Terroine, 1960) Vitamins with apparently very distinct physiological properties may be reduced, in a way, to a "common denominator," ascorbic acid

Just as ascorbic acid offers partial protection against biotin deficiency, so biotin can to a certain extent offer protection against scurvy Administered at the level of 1 mg per day to young guinea pigs throughout ascorbic acid deficiency, biotin increases the survival time It attenuates pains of the joints, paralysis of the hind quarters, and intestinal hemorrhages, but affords no protection against hemorrhage and hypertrophy of the adrenals (de Felice, 1950) Thus biotin at pharmacodynamic doses gives a definite but incomplete and temporary protection against scurvy The mechanism of its favorable action is still unknown Perhaps, it may act partly by maintaining the dehydroascorbic acid in the tissues at near normal levels (de Felice, 1954)

VII Conclusion

The known metabolic activities of biotin are varied and numerous since they extend to the metabolism of carbohydrate, lipid, protein, nucleic acid, and probably also potassium These various metabolic reactions in which biotin participates may be divided approximately into three categories synthesis, degradation, and intermediate metabolism reactions

The synthetic reactions are as follows synthesis of amino acids such as dicarboxylic amino acids, citrulline, serine, synthesis of certain purine bodies, synthesis of certain saturated and unsaturated long chain fatty acids The participation of biotin in protein or cholesterol synthesis is uncertain The catabolic reactions which require the presence of biotin are oxidation of propionic acid to succinic acid, glucose phosphorylation by hexokinase, oxidation of fatty acids, degradation of the leucine deaminated chain to acetoacetate, deamination of certain amino acids, degradation of tryptophan (contested), transamination Finally, among the intermediary metabolism reactions, the Wood Werkman reaction deserves special mention

The participation of biotin in these different reactions is of varying importance Sometimes the role of biotin in one or other of these reactions is

present in microorganisms as well as in animals, sometimes, on the other hand, it is limited to the bacterial field or to the animal field

The mechanism by which biotin is implicated in metabolic reactions is often obscure or unknown Nevertheless it is indubitable that one of the important properties of biotin is to facilitate carboxylation and decarboxylation Wood Werkman reaction, interconversion of succinic acid to propionic acid, synthesis of certain fatty acids, of citrulline, of serine, of purine bodies, degradation of the leucine deaminated chain

In all the metabolic reactions in which it participates, biotin probably plays the role of a coenzyme But the coenzyme nature of biotin has not yet been definitively demonstrated The three examples where the coenzyme role of biotin appears most clear are in relation to hexokinase in *S cerevisiae*, to oxalacetate decarboxylase, and to the enzyme which synthesizes certain long chain saturated fatty acids To be absolutely certain that biotin plays a role of coenzyme in the reactions in which it participates, it would be necessary to establish its presence as such in all the highly purified enzymes which govern the reactions in which it intervenes

If the coenzyme role of biotin is confirmed, the intimate mechanism by which the vitamin exercises this role still remains to be discovered Only one hypothesis has been formulated along these lines, according to which biotin would participate in these very different enzyme reactions through one single mechanism—that of a hydrogen carrier

Another field that is practically unexplored relates to the correlations between metabolic abnormalities and the characteristic clinical symptoms of biotin deficiency Only recently has it been suggested that certain nervous disorders may depend on a slowing of the decarboxylation of oxalacetic acid in biotin deficiency, or that alopecia might be caused by sympathetic lesions the biochemical origin of which remains to be discovered The correlations between metabolic and anatomic abnormalities in biotin deficiency remain entirely to be established

Thus, much work remains to be done to obtain further knowledge of the physiological and biochemical properties of biotin and to establish connections between them The relative ignorance which exists at present in our understanding of the properties of biotin is not exceptional, the exact mechanism of the action of thiamine or of ascorbic acid is still not definitively elucidated, although both these vitamins were known long before biotin

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Biochemistry of Vitamin E[1]

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I. Introduction

The discovery of vitamin E in the early 1920's as a fat-soluble factor necessary for reproduction in the rat has since been followed by a great many investigations of its biological distribution and action as well as its chemical structure and properties. The isolation and purification of the vitamin from the unsaponifiable fraction of wheat germ oil some thirteen years later (Evans *et al.*, 1936) was rapidly followed within two years by a number of studies describing its chemical synthesis and structural identification. The reader is referred to the review by Mattill (1954) for a summary

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of the experimental evidence leading to the chemical synthesis and identification of vitamin E

Although vitamin E has been shown to be widely distributed among plants and animals and implicated in a number of biological roles, its exact mode of action in the living cell has not yet been conclusively established. Thus far most of the studies relating to its possible functions have been based on two broad experimental approaches (1) examination of the effects of a vitamin E deficiency on whole organisms, and to a lesser extent on certain enzyme systems, and (2) examination of the effects of added vitamin E or its derivatives on isolated enzymes. The first approach has demonstrated that a deficiency of vitamin E in the diet of various animals causes or contributes to various disorders affecting a wide spectrum of tissues and organs including gonads (sterility), muscle (muscular dystrophy), nerve (encephalomalacia), epithelium (exudative diathesis), liver (massive liver necrosis), blood (hemolysis of erythrocytes), tooth (depigmentation of rat incisors), lung (hemorrhage), and kidney (degeneration of convoluted tubules) (see reviews by Mason, 1949, Mattill, 1952, Mackenzie, 1953, Sebrell and Harris, 1954, Dam, 1957). A number of deficiency studies have also suggested a possible role for vitamin E in the terminal respiratory system, in ascorbic acid synthesis and in protein and nucleic acid metabolism. The second approach dealing with the effects of added vitamin E on cell free enzymes has yielded almost completely negative results with the notable exception of a striking enhancement effect by the vitamin on the cytochrome c reductase portion of the terminal respiratory scheme in mammalian striated muscle (see reviews by Nason *et al*, 1957, Slater, 1960, Nason and Vasington, 1959, Boyer, 1960)

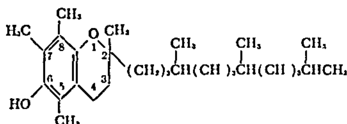
The fact that a vitamin E deficiency influences such a wide variety of animal tissues has tended to implicate its function in some basic mechanism common to most cells. To date the two most prominent possibilities for the mechanism of action of vitamin E in the living organism are (1) that it functions in a protective role as an intracellular antioxidant by preventing the oxidation of unsaturated fats and other oxygen sensitive substances such as vitamin A and ascorbic acid during storage, and/or (2) that it serves as a component, either directly as an electron carrier or indirectly possibly as a binding agent, in the cytochrome c reductase portion of the terminal respiratory chain. There is also evidence that it may be functioning in a primary role in nucleic acid metabolism and possibly in phosphorylation reactions

The object of the present article is to review and summarize critically, especially in the light of recent data, the various aspects of vitamin E metabolism with a view to evaluating its possible mechanism of action in the living cell

II Chemistry and Metabolism of Vitamin E

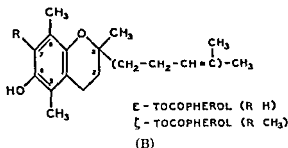
1 Chemistry

The term *vitamin E* is taken to be synonymous with the term *tocopherol* and refers to a group of mono , di , and trimethyltocols representing all seven possible methyl derivatives of the phenolic ring in the 6 hydroxy-



(α)	alpha TOCOPHEROL	5 7 8 TRIMETHYL TOCOL
(β)	beta TOCOPHEROL	5 8 DIMETHYL TOCOL
(γ)	gamma TOCOPHEROL	7 8 DIMETHYL TOCOL
(δ)	delta TOCOPHEROL	8 METHYL TOCOL
(ε)	epsilon TOCOPHEROL	5 METHYL TOCOL
(ζ)	zeta TOCOPHEROL	5 7 DIMETHYL TOCOL
(η)	eta TOCOPHEROL	7 METHYL TOCOL

(A)



(B)

Fig 1A The tocopherols

Fig 1B Newly proposed structures of ε- and ζ tocopherols from wheat (Green *et al* 1959a 1960c)

chroman nucleus containing a methyl and a particular alkyl side chain in the 2 position The structures of these seven different forms designated as α , β , γ , δ , ε , ζ , and η tocopherol are shown in Fig 1A Recent studies, however, have raised serious doubts concerning the above indicated chemical structures of ε and ζ tocopherol Evidence has been presented demonstrating that ε tocopherol from wheat bran is not 5 methyl tocol (Green *et al* , 1959a), but is instead an unsaturated analog of β tocopherol, namely 2,5,8 trimethyl 2 (4,8,12 trimethyltrideca 3,7,11 trienyl) chroman 6 ol

as shown in Fig 1B (Green *et al*, 1960c) The nature of the geometrical isomerism of the side chain which is unknown will be elucidated when ε tocopherol is synthesized These authors also conclude that ζ tocopherol from wheat is not identical with the 5,7 dimethyl tocol which occurs in rice They propose instead that it is the trimethylated member in the same series of compounds as ε tocopherol, namely 2,5,7,8 tetramethyl 2 (4,8, 12 trimethyltrideca 3,7,11-trienyl) chroman 6 ol (Fig 1B) The fat soluble and reversible oxidation reduction properties of the tocopherols may be highly significant in terms of their biological role The term vitamin E is also frequently used in a broader sense to include as well a number of tocopherol derivatives such as oxidation products and various esters when these substances exhibit biological activity Although all seven tocopherols are found in biological materials, α tocopherol is considered to be the prototype of vitamin E since it comprises about 90% of the tocopherols in animal tissue and usually displays the greatest biological activity *in vivo*

The biological activities of the different tocopherols vary somewhat depending upon the animals and assay systems used (for example, fertility test, cure or prevention of muscular dystrophy, etc) In most but not all assay systems α tocopherol has the greatest biological activity Although a number of different esters of the tocopherols including the phosphate and the monophosphate exhibit biological activity, the possibility exists that this is due to the liberation of free tocopherol by hydrolysis A number of studies indicate that biological activity in the widely used rat fetal resorption test is confined to 6 hydroxychromans or 5 hydroxycoumarins possessing a long alkyl side chain in position 2 and the —OH group in position 6 (Boyer *et al*, 1951, Karrer and Yap, 1941, Von Werder *et al*, 1939, Farber *et al*, 1953)

Although a variety of tocopherol oxidation products and derivatives including some of unknown structure have been shown to occur, our discussion here will be confined to those that have been chemically identified or implicated in biological action A reversible free radical oxidation state of α tocopherol has been indicated by the appearance of a colored product as a result of irradiation of the vitamin at liquid air temperature (Michaelis and Wollman, 1950)

Boyer (1951) isolated and tentatively characterized as an epoxide a biologically active, reversible oxidation product of *d*-α tocopherol prepared by ferric chloride oxidation in the presence of 2,2′ dipyridyl The product, designated as *d*-α tocopheroxide, is readily reduced to *d*-α tocopherol by ascorbic acid or converted to *d*-α tocopherylquinone by exposure to dilute acid Tocopheroxide has recently been shown to be a hemiacetal of α tocopherylquinone, more aptly called tocopherethoxide, and is very probably not found in biological systems (Martius and Eilingsfeld, 1957)

α Tocopherylquinone, which readily forms as an oxidation product of α tocopherol, can be reduced by ascorbic acid to the hydroquinone or reduced with cyclization to α tocopherol by ascorbic acid together with strong hydrochloric acid solution Tocopherylhydroquinone is apparently not an intermediate in the cyclization and reduction of the quinone to tocopherol Although little is known about the mechanisms of these reactions, a reasonable series of reactions have been suggested It is possible that a tocopherol free radical, a postulated carbonium cation formed from tocopherol by the loss of two electrons and a hydrogen ion, and a hypothetical hemiketal intermediate presumably arising by the addition of a hydroxyl ion to the carbonium cation are reversible stages in the oxidation of tocopherol to the quinone (Michaelis and Wollman, 1950 Harrison *et al*, 1956, Martius and Eilingsfeld, 1957, Slater, 1960) The reaction of the hypothetical carbonium intermediate with solvent alcohol should give tocopherethoxide, with hydroxyl ions it could yield the hypothetical hemiketal which would rearrange to tocopherylquinone The various interconversions are summarized in Fig 2

2 *Metabolic Conversions*

Although vitamin E is widely distributed among the tissues of various animals including man, and much is known about the variety of syndromes appearing in animals due to vitamin E deficiency (Mason, 1942, Quaife and Dju, 1949, Quaife *et al*, 1949, Sebrell and Harris, 1954), it is only recently that information has been obtained concerning the metabolic fate of this vitamin

In a study of the metabolic changes of α tocopherol, Simon *et al* (1954, 1955, 1956a) administered *d*-α tocopheryl 5 methyl C^{14} succinate by various routes to rabbits and studied its absorption and excretion Tocopherol was slowly absorbed when given orally in sesame oil, 74% of the dose appeared in the feces within 3 days, predominantly as free α tocopherol or tocopherol succinate, while very little appeared in the urine and carcass The subcutaneous administration of labeled tocopheryl succinate in oil resulted in an extremely slow absorption and a consequently slow elimination of radioactivity However, radioactivity was detected in urine within 2 hours after intravenous injection of labeled tocopheryl succinate dissolved in water with triethanolamine The fact that only 20–30% of the total dose was found in the urine after 15 days whereas 70–80% appeared in the feces indicates that absorbed tocopherol is excreted principally into the intestinal tract as postulated earlier by Klatskin and Molander (1952a, b) Over 90% of the urinary radioactivity was accounted for as metabolites of tocopherol whereas free α tocopherol made up 50% of the ethanol extractable fecal radioactivity Evidence was obtained indicating that the major urinary

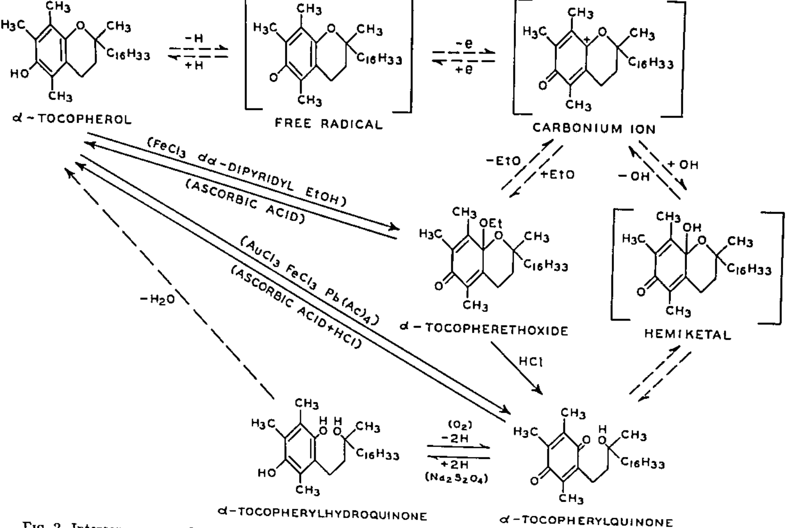


FIG 2 Interconversions of α tocopherol and its various oxidation products The chemical structures in brackets are hypothetical intermediates Dotted arrows indicate theoretical reactions solid arrows indicate demonstrable reactions and accompanying reagents

product was a "glucuronide" of a metabolic product of α tocopherol Using human subjects Simon et al (1956b) discovered two new urinary metabolites of α tocopherol, largely in conjugated form following ingestion of large quantities of vitamin E These were hydrolyzed, purified, and identified as the acid 2-(3 hydroxy 3 methyl 5 carboxypentyl) 3,5,6-trimethyl benzoquinone and its γ lactone The structure of the acid is similar to that of tocopheryl quinone except that the side chain has been decreased to three carbons containing a terminal carboxyl group The authors postulated a series of events involving hydrolytic cleavage of the chroman ring, oxidation of the terminal methyl group to a carboxyl group, and conjugation of the resulting fatty acid with coenzyme A followed by β oxidation of the C_{16} side chain In cyclization to form the γ lactone, coenzyme A would be split off from the molecule Subsequent hydrolysis of some of the lactone could account for the acidic metabolite

No radioactivity was observed in the respiratory CO_2 following oral administration of α tocopheryl succinate labeled in the 5 methyl group, whereas 0 9% and 75% of the radioactivity were found in urine and feces, respectively, after 48 hours (Niedner and Johnson 1955) Intraperitoneal injection into normal rats gave radioactive excretions of 1 7% 0 7%, and about 40% in respiratory CO , urine and feces, respectively, with essentially similar results for vitamin E-deficient rats Chromatographic studies on fecal, liver, and carcass extracts of normal and vitamin E deficient rats following intraperitoneal injections of α tocopheryl 5 methyl C^{14} succinate revealed the presence of a number of unidentified radioactive compounds, one of which may have been 2 (3 hydroxy 3 methyl 5 carboxypentyl) 3,5,6 trimethylbenzoquinone or its γ lactone (Niedner, 1957)

It is interesting to speculate that the identified urinary metabolites of tocopherol may be physiologically active forms of tocopherol Data on this point, however, are very scanty Nason and Lehman (1956) found that 2 (3 hydroxy 3 methyl 5 carboxypentyl) 3,5,6 trimethylbenzoquinone and its γ lactone were ineffective in restoring the activity of isooctane extracted DPNH and succinate cytochrome c reductases However, either derivative added *in vitro* prevented respiratory decline of oxygen consumption of histologically unaffected liver slices of rats during the latent phase of nutritionally induced necrotic liver degeneration (Schwarz *et al*, 1959) Injection of vitamin E into the portal vein immediately before removal of the liver prevents respiratory decline, but when added *in vitro*, tocopherol or its various derivatives are ineffective (Rodnan *et al*, 1956) The urinary metabolites, however, prevent this decline when added *in vitro*, the free acid being more effective than the γ lactone In addition, the fact that the lactone stimulated the initial O consumption in both deficient and normal slices with a greater effect in deficient livers led to the suggestion that the

lactone may uncouple oxidative phosphorylation or stimulate the oxidation of some other intermediate (Schwarz *et al*, 1959) A recent report, however, from the same laboratory (Corwin and Schwarz, 1960) now demonstrates an *in vitro* effect of tocopherol in preventing respiratory decline with succinate or α ketoglutarate as substrates (see Section III,2,b)

The above findings would tentatively implicate the oxidation products of α tocopherol rather than α tocopherol per se as active forms of the vitamin Experimental evidence, however, for this point of view is still lacking

Martius and Costeli (1957) found that after administration of labeled α tocopherol to rabbits two radioactive substances appeared in liver mitochondria, α tocopherol and a compound which yielded α tocopherol on acid reduction, possibly α tocopherylquinone They tended to regard the latter substance as the active form of the vitamin in tissues More recently Draper and Alaupovic (1959) indicated the occurrence in rat liver cells of two unidentified radioactive metabolites in addition to α tocopherol following intraperitoneal administration of the succinate ester of labeled tocopherol In a later communication (Draper and Csallany, 1960) they reported that the reactivation by C^{14} labeled tocopherol of isooctane extracted DPN cytochrome c reductase from a Keilin-Hartree muscle preparation is not accompanied by the enzymatic oxidation of tocopherol They found that the radioactivity in an ether extract of the reactivated enzyme system was due entirely to unchanged *d* C^{14}-α tocopherol They failed, however, to indicate the percentage recovery of radioactivity and whether or not any remained in the enzyme after the ether extraction procedure

No attempt will be made here to summarize and evaluate the biological activities of the various tocopherol derivatives in preventing and curing the various vitamin E deficiency syndromes since they have been covered in a number of reviews (see especially MacKenzie, 1953, Sebrell and Harris, 1954)

III Biological Function of Vitamin E

1 *Antioxidant Role*

One of the earliest and most frequent proposals for the mechanism of action of tocopherol has been that it serves primarily as an intracellular antioxidant, preventing the oxidation of compounds such as unsaturated fats, vitamin A, and ascorbic acid An antioxidant presumably exerts its effect by neutralizing the free radical which arises in the initial stages of an autoxidative chain reaction In its reaction with the free radical, the antioxidant also becomes an unstable free radical which undergoes irreversible changes resulting in its destruction That a free radical semiquinone form exists for vitamin E was experimentally indicated by Michaelis and Wolf

man (1950) As the tocopherols were isolated, it was clear that they exhibited antioxidant properties, and a number of investigators have attributed the wide variety of biological effects of vitamin E deficiency, at least in part, to its antioxidant action (see Mattill 1947, 1952, Mason, 1949, Mackenzie, 1953, Sebrell and Harris, 1954)

Dam and Granados (1945) were among the first to present evidence for an *in vivo* antioxidant effect of vitamin E by showing that exudative diathesis, a vitamin E deficiency disease in chicks was accompanied by a peroxidation of fat The disease, which occurs if highly unsaturated, and therefore easily autoxidizable fatty acids are present in the diet, is prevented by feeding a diet supplemented with vitamin E or free of fat, particularly unsaturated fatty acids A similar relationship between easily oxidizable fat accompanied by lipoperoxide formation and other vitamin E deficiency syndromes such as encephalomalacia in the chick, brown discoloration of the uterus and depigmentation of the incisors in the rat, and diminution of vitamin A stores in the liver of the chick and rat have been described (Dam, 1957) Other deficiency symptoms, however, such as reproductive failure in female rats, massive necrosis of the liver hemolysis of erythrocytes in rats, and muscular degeneration in the chick, may develop even in the absence of easily oxidizable fat, and are therefore attributed to factors other than to autoxidation of fat (Dam 1957) However, there are conflicting reports even on these points as indicated below

Additional support for an antioxidant role of tocopherol is the demonstration that some antioxidants and substances capable of undergoing oxidation reduction, give partial protection against certain symptoms of vitamin E deficiency, even though these compounds are unrelated to vitamin E Examples of such antioxidants and redox compounds are the synthetic substances nordihydroguaiaretic acid, Antabuse methylene blue, diphenyl *p* phenylenediamine (DPPD) and the naturally occurring substances, ascorbic acid, cystine, and others The contention that some of these compounds may substitute completely for vitamin E has been controversial, since experimental results by independent investigators has proved to be conflicting and variable, depending in part on the animal and syndrome used In the chick, they protect against exudative diathesis, encephalomalacia, and impaired growth, prevent peroxide formation in fat, and increase deposition of vitamin A in the liver (Dam *et al*, 1951, 1952) Singsen and associates (1955) have prevented encephalomalacia with DPPD at levels as low as 0 0125% of the ration Yet Scott *et al* (1955), using a severely deficient diet made up largely of *Torula* yeast, observed no protection against the signs of vitamin E deficiency (exudative diathesis) with a variety of antioxidants The deficiency was prevented by the addition of either vitamin E or brewer's yeast Similar conclusions that the prevention of

exudative diathesis does not arise primarily from the action of vitamin E as an antioxidant are indicated by the recent work of Creech *et al* (1958) Further support for the idea that the synthetic antioxidants do not replace tocopherol in its specific metabolic functions but probably simply protect it from oxidative destruction is given by the very recent report of Machlin *et al* (1959) They observed that α tocopherol prevented encephalomalacia and muscular degeneration in chicks, whereas a quinoline antioxidant prevented the first condition but not the second The antioxidant, however, was more than eight times as effective as tocopherol in preventing peroxide formation in liver homogenates and also very effective in preventing oxidative rancidity in the diet Their data, while confirming the established antioxidant action of tocopherol, also suggest a highly specific role for the vitamin In the rat, some antioxidants prevented hemolysis of erythrocytes (Christensen and Dam, 1951), depigmentation of the incisors and coloration of the adipose tissue (Aaes Jørgensen *et al*, 1951), massive hepatic necrosis and lung hemorrhage (Dam and Granados, 1951) They also caused an increase in liver stores of vitamin A (Dam *et al*, 1952) and tended to prevent sterility (Dam and Granados, 1952) Moore and Sharman (1959) found that dietary DPPD corrected lability to hemolysis by dialuric acid in rats which had suffered from prolonged deprivation of vitamin E

Muscular dystrophy was partially prevented by synthetic antioxidants in vitamin E deficient rats and rabbits (Moore *et al*, 1954, Draper and Csallany, 1958), calves (Blaxter *et al*, 1953), and lambs (Draper and Johnson, 1956) In guinea pigs, DPPD was observed to delay but not prevent the occurrence of muscular degeneration (Shull *et al*, 1957, 1958)

Although methylene blue and DPPD were earlier reported to be effective in correcting reproductive failure in vitamin E deficient female rats (Dam and Granados, 1952), Christensen *et al* (1956) in Dam's laboratory subsequently found that methylene blue was completely ineffective in diets when thoroughly freed of tocopherol, thus suggesting that the antioxidant may function by sparing residual tocopherol levels otherwise insufficient for protection The results of Draper *et al* (1958) were attributed to this view DPPD prevented fetal resorption in rats, but this protection completely disappeared in the third generation, suggesting that the antioxidant may be protecting the small amounts of residual tocopherol, which eventually disappear Moore and associates (Moore *et al*, 1954, Sharman and Moore, 1958) also failed to affect reproduction with these antioxidants, although they were able to protect against brown coloration of the uterus and against tubular degeneration

Tappel (1953, 1954), in demonstrating that the oxidation of unsaturated fatty acids catalyzed by hematin compounds could be inhibited by α

tocopherol and other antioxidants, has suggested that a mechanism of action of vitamin E may involve *in vivo* inhibition of hematin catalysis The observation that alcohol dehydrogenase is inhibited in the presence of metal ions by low concentrations of α tocopherol (2 7 × 10^{-6} *M*, 50% inhibition) and the antioxidant nordihydroguaiaretic acid (2 7 × 10^{-7} *M*, 25% inhibition) may be of biological significance (Tappel and Marr, 1954) Tappel and Zalkin (1959) and Zalkin and Tappel (1960) in claiming a significant inhibition of lipid peroxidation by added tocopherols in isolated mitochondria and microsomes of rat liver and rabbit heart postulated that peroxidation damage to these cellular structures was responsible for the metabolic derangements leading to the gross symptoms of vitamin E deficiency They suggested that vitamin E functions primarily by inhibiting lipid peroxidation Their data, however, are open to definite criticism The results with liver and kidney supposedly demonstrating a significant difference in peroxidation products (as measured by the thiobarbituric acid reaction) between the control and vitamin E deficient animals (Zalkin and Tappel, 1960) show in fact an overlap of the standard deviations between the two groups In addition there is a relatively high level of thiobarbituric and reactants in the tissues from control rabbits which they admit is the major difficulty in using the method to measure lipid peroxidation products accumulating in tissues of vitamin E deficient animals In fact the mitochondria, microsomes, and soluble cytoplasm account for only one tenth of the thiobarbituric acid reactants in fractionated liver Most of it is in the cell debris which shows no significant difference between the vitamin E deficient and control groups Finally, the muscles from dystrophic animals, although the first tissues to display signs of a vitamin E deficiency, show no significant difference as compared to the controls in accumulating peroxidation products Of some significance is the serious limitation of the thiobarbituric acid procedure itself which is supposed to measure malonaldehyde formed in lipid peroxidation and is admittedly not highly specific when applied to whole animal tissues Tappel and Zalkin (1960) ascribed their findings that isolated microsomes from vitamin E deficient rabbits had sixfold as much peroxidation products per milliliter as that from controls to an *in vivo* peroxidation effect These results must be regarded with some skepticism in view of the author's admission that the microsomes of the vitamin E deficient animals were more densely packed and contained six times as much nitrogen as that of the controls If the data had been expressed on a nitrogen basis, instead of on a volume basis, there would be no difference in the level of peroxidation products between microsomes of the vitamin E deficient rabbits and the controls Bieri and Anderson (1960) observed that *incubation of homogenates* of cer

tain tissues (e g , liver) of vitamin E deficient animals show greater peroxidation product formation than that of control animals, a phenomenon that varies widely with different tissues and different animal species Pritchard and Singh (1960) found that the tissue peroxide content and susceptibility to peroxidation increased on diets low in vitamin E and that in brain and heart the most active subcellular area for lipid peroxide formation was located in the microsomal supernatant solution This is contrary to the results of Tappel and Zalkin (1959) who indicated that of the various tissues examined only the liver showed significant difference in the accumulation of lipid peroxidation products between vitamin E deficient and control rabbits The results of Tsen and Collier (1960a) indicated that erythrocytes from tocopherol deficient rats do not have an increased level of lipid peroxides until after treatment with an oxidizing agent such as dialuric acid There was no apparent relationship between susceptibility to hemolysis in erythrocytes from tocopherol deficient and tocopherol supplemented rats and the level of erythrocyte glutathione (Tsen and Collier, 1960b) Certain substances (e g , selenite, iodoacetate) decreased the glutathione content of red blood cells to very low levels with little accompanying hemolysis, while other substrates could cause complete hemolysis with little or no decrease in levels of erythrocyte free glutathione Bieri and Pollard (1959) have contended that the condition of exudative diathesis in chicks is probably caused by dietary conditions leading to the formation of peroxides in tissues The fact that either vitamin E or selenium can prevent this condition provides an argument in favor of a more specific role for tocopherol other than as nonspecific antioxidant since the mechanism of action of selenium must be other than that of antioxidant (see Section IV,2) The observation by Bieri (1959) that dietary selenium and cysteine significantly reduce peroxidation in certain tissues of vitamin E deficient chicks although they are without effect *in vitro* further complicates the picture The possibility that selenium is acting by sparing tocopherol seems unlikely in view of the report by Bieri *et al* (1958) that the rate of depletion of vitamin E is not influenced by biologically active selenium That the *in vitro* formation of peroxides may not be indicative of *in vivo* formation is reported by Carpenter *et al* (1959) who found that the presence of tocopherol in the diet made no difference in the peroxides *in vivo* of whole liver and testis of rats, in contrast to the opposite claims made by Tappel and his associates and by Bieri's group already discussed above The important point that tocopherol exerts a protective action against *in vivo* formation of peroxides is therefore highly controversial

The possible antioxidant role of tocopherol in the terminal enzymatic step of ascorbic acid biosynthesis is discussed more fully in Section III,2,C

It should be emphasized that no attempt has been made here to cover all the literature on the antioxidant action of vitamin E Instead, the plan has

been to discuss some representative recent findings along lines that reflect the present status of the problem. In brief the evidence is convincing that part of the nutritional value of vitamin E is due to its antioxidant activities. Whether or not this is its primary role, if such a viewpoint can be taken, is controversial. The deficiency studies also definitely indicate that vitamin E is functioning as well in a specific capacity in addition to its nonspecific antioxidant role. This is especially indicated by experiments in which severely deficient diets were used (Scott *et al.*, 1955, Christensen *et al.*, 1956). Antioxidants failed to be effective, suggesting that they were acting by sparing or protecting small amounts of vitamin E present in the diet or tissues, thereby delaying the onset of deficiency symptoms. Thus, Shull *et al.* (1957, 1958) merely delayed the onset of muscular dystrophy with DPPD, and tissues of animals on vitamin E deficient diets which also contain methylene blue showed higher values for tocopherol than tissues from animals on similar diets without methylene blue (Dam *et al.*, 1951, Markees, 1953).

The evidence that vitamin E may be functioning as a component in the terminal respiratory system is discussed in a later section (p. 61). Finally the recent report by Pollard and Bieri (1959c) and Bieri *et al.* (1960) that normal chickens can be raised on tocopherol free diets and that their tissues contain no detectable tocopherol would tend to refute all the previous nutritional work demonstrating a specific action of tocopherol. It would also imply that tocopherol cannot be regarded as a vitamin. Further experimental examination of this point will be awaited with interest, especially in view of the problems in properly extracting and separating tocopherol as discussed in Section III,2,c,i below.

2. *Metabolism and Electron Transport*

a. Effect of Dietary Vitamin E Deficiency on Oxygen Consumption. Examination of the numerous papers describing a marked increase in the oxygen consumption of muscle strips and homogenates from vitamin E deficient animals (Victor, 1934, Madsen, 1936, Friedman and Mattill, 1941, Houchin and Mattill, 1942a, b, c, Houchin, 1942, Kaunitz and Pappenheimer, 1943, Morgulis and Jacobi, 1946, Hummel and Basinski, 1948, Markees, 1948, Roderuck *et al.*, 1949, Hummel and Melville, 1951, Rosenkrantz, 1955) reveals that in many of these reports the data are somewhat diffuse and inconsistent, having been obtained in a number of cases with animals in extreme deficiency and on the verge of death. Although the data in general indicate a definite tendency toward an increased respiration in vitamin E deficient muscle tissue, the range of oxygen uptake values for control and deficient preparations frequently overlap or the conditions of assay are so specialized as to cast doubt on the significance of the observed differences. For example, in order to demonstrate differences

in oxygen consumption between skeletal muscle preparations from control and vitamin E deficient rabbits, added calcium ions were necessary (Rosenkrantz, 1955) The fact that calcium caused a marked inhibition (approximately 60%) of respiration in both sets of tissues, that an overlapping of

TABLE I

EFFECT OF VITAMIN E DEFICIENCY ON OXYGEN UPTAKE OF VARIOUS TISSUES

Reference	Animal	Tissue	Treatment	Q_{O}
Rosenkrantz (1955)	Rabbit	Skeletal muscle slices	Normal	4 17 ± 0 56
			E deficient	4 19 ± 0 59
		Skeletal muscle slices plus added Ca^{++}	Normal	1 67 ± 0 48 (0 99–2 39)
			E deficient	2 48 ± 0 49 (1 76–3 31)
Hummel and Melville (1951)	Rabbit	Muscle strips	Normal	1 11 (0 49–1 88)
			E deficient	1 86 (1 74–1 94)
Friedman and Mattill (1941)	Rat	Muscle strips	Normal	
			1 hour	2 19
			2 hours	1 99
			3 hours	1 80
			4 hours	1 70
			E deficient	
			1 hour	2 52
			2 hours	2 17
			3 hours	2 03
			4 hours	1 79
Victor (1934)	Duck	Adductor muscle	Normal	3 0 5 7 4 7
			E deficient	5 4 3 1 5 6

data still occurred between the control and deficient preparations, and that the oxygen uptake in both sets was the same in the absence of calcium raises some question about the significance of these results It is also interesting to note that although Houchin (1942) observed a 60% increase in oxygen consumption by vitamin E deficient slices, the mince and homogenates of these tissues gave values identical for normal and dystrophic muscle Table I shows a compilation of some of the data reported in the literature illus

trating the variability of oxygen consumption in vitamin E deficient muscle The *in vivo* administration of vitamin E usually caused a lowering of oxygen consumption compared to that of normal muscle but it generally had no effect when added to *in vitro* systems

Apparently not all tissues responded alike in vitamin E deficient animals Kaunitz and Pappenheimer (1943) observed no effect of vitamin E deficiency on the oxygen uptake of rat liver, and Houchin (1946) observed an increased oxygen consumption of vitamin E deficient hamster kidneys Rosenkrantz (1955) found that rabbit adrenal cortex and liver slices showed a definite increase in oxygen consumption whereas heart and kidney remained unchanged His observation that an increased oxygen consumption by adrenal cortex could be elicited by other vitamin deficiencies and stress situations strongly hints at the nonspecificity of vitamin E deficiency in stimulating respiration

b Effect of Dietary Vitamin E Deficiency on Oxidative Enzyme Systems A variety of papers, including those of a conflicting nature, are present in the literature relating to the effects of a vitamin E deficiency on different oxidative enzyme systems Among the earliest of these reports was that by Houchin and Mattill (1942a, b) describing a two to threefold increase in succinoxidase activity by hamster dystrophic muscle Basinski and Hummel (1947) were subsequently unable to demonstrate any difference in succinic dehydrogenase activity of muscle homogenates from either dystrophic hamsters or hamsters receiving an α tocopherol acetate dietary supplement Jacobi *et al* (1950b) observed no changes in malic dehydrogenase, cytochrome oxidase, succinic dehydrogenase, lactic dehydrogenase, fumarase, and adenosinetriphosphatase activities of muscle homogenates from rabbits suffering with incipient muscular dystrophy Shirley *et al* (1956) however, reported an increase in the succinoxidase of heart and skeletal muscle and liver from *vitamin E supplemented* animals as well as an increase in liver lactic dehydrogenase Rosenkrantz (1958) found a decrease in the activity of ketoglutaric dehydrogenase and isocitric dehydrogenase in the dystrophic muscle of rabbits in the terminal stages of the disease The report of Aloisi (1955) of a decrease in cytochrome oxidase activity of dystrophic muscle from vitamin E deficient guinea pigs is opposed by the recent claim of Allen *et al* (1958) that an increase occurs in cytochrome oxidase (and cytochrome reductase) activity in leg muscle of vitamin E deficient rabbits The variability of their cytochrome reductase data (Allen *et al* 1960) and the fact that they were obtained during the terminal stages of the deficiency disease cast doubt on the significance of their results Corwin and Schwarz (1959) found that liver mitochondria of vitamin E deficient rats showed a substantial decline in succinate oxidation in the presence of added diphosphopyridine nucleotide (DPN) during the

second half hour of measurement as compared to mitochondria from vitamin E supplemented animals The decline, which was attributed by them to an inhibition of succinate oxidation by accumulated oxaloacetate, was reversed within 30–40 minutes after intraportal injection of α tocopherol, but not by addition *in vitro* The DPN cytochrome c reductase activity in mitochondria and microsomes was unaffected by the vitamin E deficiency Corwin and Schwarz (1960) have now found that liver homogenates of vitamin E deficient rats experience a marked decline of oxygen consumption with α ketoglutarate and succinate as substrates This striking decrease, which occurs during the 60 to 90 minute period of measurement of oxygen uptake, is completely prevented by dietary vitamin E (dietary selenium is without effect) or by the *in vitro* addition of α tocopherol In effect, these results constitute the first evidence for the impairment of the enzymatic processes of succinate and DPNH oxidation by an *in vivo* vitamin E deficiency which can be reactivated by the *in vitro* addition of the vitamin It fits in well with the evidence from the present author's laboratory (Nason and Lehman, 1956, Donaldson *et al*, 1958b) that vitamin E is a component of mammalian DPN- and succinate cytochrome c reductases Corwin and Schwarz (1960) also achieved an *in vitro* prevention of the decline of α ketoglutarate and succinate oxidation in vitamin E deficient rat liver homogenates by addition of the tocopherol metabolite of Simon *et al* (1956b), menadione, or DPPD The possibility must be considered that the latter three substances, which are all capable of reversible oxidation reduction reactions, are acting in the *in vitro* system as artificial electron carriers and thus are probably bridging a possible electron transport gap resulting from a tocopherol deficiency The recent data of Pollard and Bieri (1959c) also indicate that the DPN cytochrome c reductase activity of heart muscle preparations from vitamin E deficient chicks is generally lower although not significantly different from that of normal chicks

c Effect of Dietary Vitamin E Deficiency on Other Enzymes A vitamin E deficiency has been reported to cause changes in the activity of various enzymes in addition to those already mentioned in previous sections Barber *et al* (1949) and Roderuck *et al* (1949) observed a significant decrease in the aspartic glutamic transaminase activity of skeletal muscle homogenates from dystrophic guinea pigs and rabbits, a similar decrease as well as a lowered content of pyruvic and ketoglutaric acid in vitamin E-deficient rabbit muscle were also reported by Barbiere and Zerli (1958) Olson and Dinning (1954), however, found no change in transaminase activity of liver from rats maintained on a vitamin E deficient, necrotic liver producing diet Other enzyme activities that have been reported to decrease in vitamin E deficient tissues include acid and alkaline phosphatases (Smith and Nehorayan, 1958), cholinesterase (Bloch, 1942, Stoerk and Morpeth, 1944,

Hess and Viollier, 1948, Collier and Dellert, 1953), plasma lipase (Hess and Viollier, 1948), and kidney transamidinase (Van Pilsum and Wahman, 1960) Those that have been found to increase are betaine homocysteine transmethylase (as well as vitamin B_{12} content) (Giacalone *et al*, 1956, Rabbi *et al*, 1956), aldolase (Beckmann and Buddecke, 1956), dipeptidase (Weinstock *et al*, 1956), and xanthine oxidase (Dinning, 1952a,b, 1953, Olson and Dinning, 1954)

A decreased lactate production from fructose 1,6 diphosphate (Hummel, 1948), a lowered synthesis of ascorbic acid (McCay and Carpenter, 1958), and an increased incorporation of formate into certain amino acids and bases have also been associated with vitamin E deficiency During the past two years there has been a rapid succession of papers implicating vitamin E in the biosynthesis of ascorbic acid The observation that enzyme preparations from livers of vitamin E deficient rats and rabbits synthesize 70 to 90% less ascorbic acid than those of control animals (Caputto *et al*, 1958) were soon followed by findings that not only tocopherol but other substances such as Versene and salts of Co^{++}, Mn^{++}, and Ce^{+++} independently reactivated the *in vitro* synthesis of ascorbic acid by liver preparations from vitamin E deficient animals Moreover, these substances also stopped the production of presumed peroxidation products as measured by the thio barbituric acid In addition to the above findings (McCay *et al*, 1959, Kitabchi *et al*, 1959 Carpenter *et al*, 1959), these workers also showed that the impaired synthesis of ascorbic acid was due to the inhibition of the microsomal enzyme system, gulonolactone oxidase, which mediates the oxidation of gulonolactone to yield ascorbic acid Although they demonstrated that the most abundant thiobarbituric acid reacting material produced in the process of peroxidation of microsomal lipid was malonaldehyde, they concluded that malonaldehyde was not the factor responsible for the inhibition of ascorbic acid synthesis They suggested that gulonolactone oxidase is inhibited, either in the intact microsome or after solubilization, by some factor, possibly a free radical related to the formation of malonaldehyde but not by malonaldehyde itself (Kitabchi *et al*, 1960) Presumably tocopherol and other substances which reinstate ascorbic acid synthesis act by destroying or preventing the formation of this unidentified inhibitor

Of particular interest is the work of Dinning and associates (Young and Dinning, 1951, Dinning 1955, Dinning *et al* 1955 1956, Dinning and Day, 1957a, b) suggesting a primary function for vitamin E in the regulation of nucleic acid turnover The findings by these workers that vitamin E deficient animals display an increased allantoin excretion, an increased liver xanthine oxidase, and incorporation of more formate and less glycine into nucleic acids and proteins have led them to suggest that vitamin E may be regulating an exchange reaction involving the number 2 carbon of the

purine ring, possibly by affecting coenzymes derived from folic acid That the increased incorporation of formate [primarily in the deoxyribonucleic acid (DNA) fraction] as a result of a vitamin E deficiency was especially noted in skeletal muscle and bone marrow is significant since these two tissues are severely affected in the vitamin deficient rabbit (Dinning, 1952a, b, Dinning and Day, 1957a, b, 1958) An increased P^{32} incorporation into nucleic acids of various tissues was also reported in vitamin E deficient rabbits (Dinning *et al*, 1956) Diehl (1960) reported a sharply increased incorporation of labeled glycine into the skeletal muscle protein of vitamin E-deficient rabbits Other recent papers describing the effects of vitamin E deficiency on the metabolism and composition of various tissues include observations of a marked decline in muscle myoglobin (Schottelius *et al*, 1959), an increase in collagen nitrogen as well as a decrease in contractile protein nitrogen (Bender *et al*, 1959), an increased 1 methyl histidine excretion and decreased anserine synthesis in muscle (McManus, 1960), and the presence of an unidentified reducing substance whose concentration seems to be directly related to the level of vitamin E or other antioxidant in the diet (Crider *et al*, 1960)

An increased RNA and DNA content in vitamin E deficient animals has been shown by a number of investigators (Joseponits *et al*, 1957, Antoni *et al*, 1958, Azzone and Aloisi, 1958), although Niesar (1957) found that infusion of tocopherol into rats also caused an increased nucleic acid content in various tissues

It is still too early to evaluate the significance of the different metabolic and enzymatic changes indicated above in terms of a primary role for vitamin E Certainly one of the most promising leads is the apparent involvement of tocopherol in nucleic acid metabolism as suggested by the work of Dinning and his colleagues

d Effects of Added Vitamin E on Cell Free Enzymes In most studies of the *in vitro* effects of vitamin E on enzyme systems the vitamin was added as α tocopheryl phosphate in view of its greater water solubility as well as the implication of an active form of the vitamin by analogy with other biological phosphate compounds In virtually all cases, the addition of α tocopheryl phosphate markedly inhibited the different enzymes tested including succinoxidase and succinic dehydrogenase (Govier *et al*, 1945, Houchin, 1946, Ames, 1947a, b, Basinski and Hummel, 1947, Jacobi *et al*, 1947, 1950a, Zierler *et al*, 1948, Schmitz, 1950, and Rabinowitz and Boyer, 1950), lactic dehydrogenase (Govier *et al*, 1946), DPNases (Spaulding and Graham, 1947, Govier and Jetter, 1948), liver acid phosphatase (Jacobi *et al*, 1947), hyaluronidase (Miller and Desert, 1949), liver esterase (Van Der Meer and Nieuwerkerk, 1951), and lipoxidase (Holman, 1947, Kunkel, 1951) Some of the suggestions to explain the inhibitory effect of added

tocopheryl phosphate on cell free enzymes have invoked (1) its binding action on calcium thus presumably inhibiting succinoxidase by preventing the breakdown of DPN by the supposed inhibition of DPNase (Govier et al, 1946, Ames et al, 1947a, b) and (2) its nonspecific protein binding action (Ames and Risley, 1949) However, the most likely mechanism of inhibition by tocopheryl phosphate has been indicated by Rabinovitz and Boyer (1950) They concluded from their studies in conjunction with other data reported in the literature that the inhibitory effects of α tocopheryl phosphate are related not to its vitamin properties but rather to its nonspecific surface active or detergent properties as an anion with a large polar group The effect of α tocopheryl phosphate and α tocopheryl succinate in replacing an extractable factor from purified soluble pyruvate oxidase of an acetate requiring *Escherichia coli* mutant reported by Hager (1957) is probably due to a surface action effect on the enzyme

e Possible Cofactor Role of Vitamin E at the Enzymatic Level i Presence of tocopherol in electron transport enzyme systems, and limitations of the tocopherol assay Bouman and Slater (1956 1957) were the first to report the presence of vitamin E in active respiratory enzyme systems They showed that Keilin Hartree horse heart muscle preparations contained 0 3–0 4 μmole of α tocopherol per gram of protein and demonstrated an additional 1–2 μmoles upon reduction of the extracted lipid with ascorbic acid in hydrochloric acid They found vitamin E to be the only fat soluble vitamin present in the heart muscle preparation in appreciable amounts Considerably higher tocopherol values were obtained by Donaldson and Nason (1957) for a corresponding enzyme system from rat skeletal muscle and bovine heart muscle with most of the tocopherol concentration also appearing after employing the ascorbic acid HCl reduction method of Harrison et al (1956) Part of the explanation for the discrepancy, aside from possible species and nutritional differences, may be in the incomplete extraction of vitamin E as well as in the difficulties of separating it from other reducing substances before determination as indicated below Bouman and Slater (1956, 1957) extracted their lyophilized preparations for 4 hours with ethanol whereas Donaldson and Nason (1957) extracted trichloroacetic acid treated preparations with a more efficient petroleum ether ethanol mixture for 12–24 hours It has been observed that treatment of extracted lyophilized bovine heart DPN cytochrome c reductase with trichloroacetic acid released significantly more tocopherol upon further extraction (Vasington and Nason in preparation)

The recent finding by Bouman et al (1958) that the ascorbic acid HCl reduction product of coenzyme Q or ubiquinone (which closely resembles α tocopherol but is not identical with it) has a similar absorption spectrum and an R_f almost indistinguishable from that of α tocopherol on the $ZnCO_3$

paper chromatogram seriously limits the usefulness of this reduction method with the above widely utilized chromatographic assay for tocopherol Bouman *et al* (1958) reported that the coenzyme Q content of heart muscle preparations (approximately 2 5 µmoles per gram protein) accounts for the so called increased "tocopherol" content obtained by acid reduction Oddly enough as supporting evidence, their claim that oxidation of the acid reduction product yielded the spectrum of ubiquinone (peak at 276 mµ) and not α-tocopherylquinone (peaks at 261 mµ and 269 mµ) is in direct contradiction to their original findings Originally Bouman and Slater (1956) identified the tocopherol of a Keilin Hartree muscle preparation (isolated by fractionation and chromatographic procedures following ascorbic acid HCl reduction) as authentic tocopherol since it showed the typical two banded spectrum of tocopherylquinone (262 mµ and 269 mµ) as a result of oxidation No mention was made then of a 276 mµ peak following oxidation despite the fact that virtually all the tocopherol appeared as a result of the ascorbic acid HCl reduction procedure Pudelkiewicz and Matterson (1960) observed that coenzyme Q interfered in the analysis of tocopherol if it was allowed to come in contact with a reducing substance

Edwin *et al* (1960) in critically examining the existing assays for vitamin E concluded that many of the literature values for tocopherol contents in animal tissues "must be seriously in error " They emphasized that the two major problems in the determination of tocopherol revolved about (a) a suitable extraction procedure without loss of the vitamin which is sensitive to oxidation, and (b) the separation of the vitamin from large quantities of other reducing substances prior to its determination As a result of extensive experimentation they recommended a sequence of detailed procedures including the freezing and grinding of tissues with a mixture of Na_2SO_4 and acetone at $-70°$, acetone extraction, and a number of other steps before applying two dimensional chromatography In failing to confirm the experiments of Bouman and Slater (1957) who contended that both darkness and an antioxidant are necessary for the quantitative recovery of vitamin E during its heating in ethanol, they suggested that the results of these workers were due to an ineffectual extraction procedure Edwin *et al* (1960) have also claimed that vitamin E values obtained without the use of paper chromatographic separation may be grossly misleading because nontocopherol reducing impurities are usually present in tissues in amounts much greater than tocopherol itself They implied that this is the case for the results of Draper and Csallany (1958) who on the basis of nonchromatographic procedures reported neither a change in tocopherol levels of rabbit muscle and rat liver by dietary depletion nor a modified depletion rate of the vitamin from the liver by the administration of DPPD to vitamin E deficient animals

The detailed report by Bieri *et al* (1960) that chicks raised to maturity without dietary vitamin E are apparently normal but devoid of the vita

min would seem to reflect seriously on the status of tocopherol as a vitamin by present standards Judgment of these results must be held in reserve, however, in view of the above questions raised by Edwin *et al* (1960) with regard to the uncertainties of the tocopherol assay In addition it should be noted that the extremely small quantities (2-4 gm) of *vitamin E depleted* tissues used by Bieri *et al* (1960) for analysis would considerably lessen the chances of detecting tocopherol It has been the present authors' experience that at least 20 gm of *normal* tissue are necessary in order to do a reliable tocopherol assay by the paper chromatographic procedure It is logical to expect that considerably more tissue from vitamin E depleted animals would be needed for a reliable degree of certainty as to whether or not tocopherol is present While Bieri *et al* (1960) admit that small but definite quantities of tocopherol might have been provided as contaminants in the vitamin E-depleted diets they assume that autooxidation of unsaturated fatty acids in the digestive tract and in the body tissues would have effectively destroyed this amount of tocopherol They concluded that "no more than 0 002 μmole of tocopherol/gm of tissue" is present in vitamin E depleted chick heart By comparing this tocopherol value, oddly enough to the tenfold higher value for cytochrome c in *normal* rat heart muscle they implied that it is therefore of no significance in the chick They failed to consider however, that the presence of high levels in their experimental diets of methionine or cystine might have a marked tocopherol sparing effect, as subsequently reported by Schwarz and Foltz (1960)

Colishaw *et al* (1957) reported that the mitochondrial fraction of chicken liver homogenate contained the highest concentration of α tocopherol These analyses were performed without the use of a reduction procedure Colishaw and associates found, however, that reduction with $SnCl_2$ in HCl gave no apparent increase in tocopherol values, implying that either chicken liver mitochondria contain no ubiquinone or the reduction procedure warrants still further study Crane *et al* (1959) observed that particulate, submitochondrial electron transport preparations contain in addition to coenzyme Q approximately 1 μmole of α tocopherol per gram protein Nason and Vasington (1959) have purified a bovine heart muscle DPNH cytochrome c reductase 300 fold and have found α tocopherol to be present in all fractions, including the most purified preparation, as determined independently by the paper chromatographic methods of Green *et al* (1955) and Lester and Ramasarma (1959) In view of the interference by coenzyme Q, previous reduction by ascorbic acid HCl was not employed in this assay for α tocopherol In some cases, but not all, the concentration of tocopherol ranging from 0 1 to more than two μmoles per gram protein was greatest in the most highly purified fraction Harris and Mason (1956) found the highest concentration of tocopherol in the mitochondria, a moderately high level in the microsomes, and a relatively low quantity in the nuclear fraction Dr Y Hatefi of the Enzyme Institute recently found that highly

purified preparations of a particulate DPN cytochrome c reductase from beef heart mitochondria contained significant concentrations of α tocopherol approximately 2 μmoles per gm protein (personal communication) Finally, Slater (1960) has definitely found that tocopherol is localized in the sarcosomes (mitochondria) of horse heart and ox heart muscle preparations

Joel *et al* (1958) made an extensive study of the lipid components of a succinate and DPNH-oxidase system derived from the mitochondrial membranes of beef heart muscle They did not report the presence of any α tocopherol, but did find coenzyme Q Marinetti *et al* (1957a, b) were not able to find tocopherol in the lipids of cytochrome oxidase nor in a purified pig heart cytochrome b cytochrome c preparation free of cytochrome oxidase

Of some interest is the report of Green *et al* (1959b) that of a number of microorganisms grown in deep culture, only those containing chlorophyll also contained α tocopherol

ii Role of tocopherol in the DPN- and succinate cytochrome c reductase systems (1) Effect of aging and organic solvent extraction The various chemical studies demonstrating reversible oxidation products of tocopherol have served as a basis for suggesting that the vitamin may be functioning in biological oxidation reduction reactions other than as an antioxidant The work of Nason and co workers during the last four years has indicated that vitamin E may act at the enzymatic level in the cytochrome c reductase portion of the terminal respiratory chain Nason and Lehman (1956) initially observed that the addition of α tocopherol to partially purified particulate preparations of rat skeletal muscle increased the rate of DPNH oxidation two to sixfold by specifically restoring cytochrome c reductase activity The fact that it was necessary to store the muscle fraction at −15°C for 1–2 weeks with daily thawing in order to demonstrate the tocopherol enhancement effect suggested a possible dissociation of a lipid substance, perhaps α tocopherol or a related compound, from the enzyme—a process that might be more efficiently accomplished by extraction of the enzyme with a nonpolar solvent They found that extraction of the muscle fraction with isooctane (2,2,4 trimethylpentane) resulted in a 75–95% decrease in DPN cytochrome c reductase activity accompanied by removal of only 10% of the total lipid Activity was restored by the addition of tocopherol or *d*-α tocopherethoxide whereas tocopheryl esters, other fat soluble vitamins, various cofactors, steroids, fats, fatty acids, and a number of antioxidants showed no effect The crude lipid residue from the isooctane extract, however, which apparently contained no free tocopherol was at least five times as active as tocopherol on a weight basis

Morrison *et al* (1956) and Marinetti *et al* (1958) also demonstrated a restoration of isooctane extracted heart muscle preparations not only by tocopherol, but by hemin lipid fractions from cytochrome oxidase and by

long chain lipid esters containing no vitamin E The claim by Deul et al (1958) that added α tocopherol also restores the cytochrome oxidase (as well as cytochrome c reductase) of isooctane extracted Keilin Hartree heart muscle preparations is contrary to the independent findings of Nason and Lehman (1956), Morrison et al (1956) and Igo et al (1959)

Subsequent experiments by Donaldson et al (1958b) demonstrated that although isooctane extraction of particulate preparations of rat skeletal muscle and bovine heart muscle removed only 10% of the vitamin E present, almost complete inactivation of the cytochrome c reductase occurred The concentration of vitamin E in the lipid residue obtained by isooctane extraction was shown to be too low to account for restoration of the enzyme The isolation and identification of the active substance in the residue as a mixed triglyceride with stearate oleate, and palmitate components (Donaldson et al, 1958a) prompted an examination of other substances including various glycerides esters and natural products Of a total of more than 100 different compounds tested, about fifteen natural and synthetic products including butter oleomargarine, and n butyl stearate also reactivated cytochrome c reductase Since the isooctane extracted enzyme still contains about 90% of its original vitamin E in a presumably bound form, it was suggested that the addition of the above active lipids indirectly reactivate the enzyme by displacing bound tocopherol thus making it available to the active sites of the enzyme In support of the hypothesis Donaldson et al (1958b) showed that only those lipids which restored cytochrome c reductase potentiated the removal of vitamin E by subsequent isooctane extraction They also found that by aging the rat skeletal or bovine heart muscle enzyme fractions under given conditions in order to dissociate more of the endogenous tocopherol (with or without isooctane extraction) it was possible to restore cytochrome c reductase activity specifically by the tocopherols Other substances previously active, such as the mixed triglyceride n butyl stearate butter and oleomargarine, were now not only ineffective but also failed to potentiate the removal of vitamin E by subsequent isooctane extraction Unfortunately, the aging effect in producing a tocopherol specific enzyme has proved to be an unpredictable phenomenon occurring with some preparations but not with others

The wide divergence in experimental data obtained by different workers in utilizing the technique of extracting enzymes with organic solvents (e g isooctane) as a means of elucidating lipid requirements calls for extreme caution in interpretation of results The suggestion by Deul et al (1958) that inactivation by isooctane extraction is caused by a physical disruption of the respiratory chain or by adsorption of isooctane on the enzyme surface and that tocopherol reactivates by restoring the physical structure or by dissolving the isooctane seems to apply in some cases but not in others It appears to depend upon the enzyme preparation, the conditions of solvent

extraction, and undoubtedly other unknown factors Pollard and Bieri (1958, 1959c) originally reported that the decrease in DPNH oxidase and DPN cytochrome c reductase activities of chick heart and rat skeletal muscle preparations extracted or homogenized with isooctane could be restored not only by α tocopherol or vitamin K_1, but simply by centrifuging (10,000 g for 15 minutes) or lyophilizing the treated enzymes and reconstituting to the original volume All attempts by Vasington and Nason (manuscript in preparation) using various particulate and digitonin solubilized fractions of rat skeletal muscle and bovine heart muscle in approximately twenty different experiments have failed to confirm the work of Pollard and Bieri relating to isooctane inhibition The addition of α tocopherol to isooctane treated enzymes restored DPN and succinate cytochrome c reductase activity whereas lyophilization was unsuccessful In virtually all experiments tried, centrifugation as high as 144,000 g for 1 hour also failed to reactivate In the few experiments where activity was restored by centrifugation, a second extraction with isooctane yielded preparations that were now restored only by α tocopherol and other lipids, but not by subsequent high speed centrifugation These results were communicated to Pollard and Bieri who then found (Pollard and Bieri, personal communication, 1959a, b) that reactivation in all cases of chick heart DPNH oxidase and in approximately 50% of their experiments with beef heart DPN cytochrome c reductase could not be effected by centrifugation although tocopherol did restore activity Their claim that restoration of isooctane treated beef heart enzyme by centrifugation occurs only with fresh preparations is at direct odds with the work of Nason and his associates, who were consistently unable to lower cytochrome c reductase activities unless the rat skeletal or bovine heart muscle preparations were aged for at least 1 or 2 days before extration with isooctane Draper and Csallany (1960) in contradiction of Pollard and Bieri (1958, 1959c) observed that successive centrifugation of isooctane extracted Keilin Hartree preparations resulted in an even greater inactivation of DPN cytochrome c reductiase instead of a reactivation

The complex and unclarified nature of the effects of nonpolar solvents on enzyme system and the pitfalls of extrapolating these results from one set of conditions to another are further illustrated by the recent data of Pollard and Bieri (1959b) Their concession that the technique of homogenizing with solvents "gave more reproducible results than the extraction technique," their data showing that hexane treated chick heart and aged beef heart preparations are restored by centrifugation whereas isooctane treated preparations essentially are not, and their experiments demonstrating that inhibition of DPN-cytochrome c reductase by isooctane treated cytochrome c is reversed by tocopherol or centrifugation in the case of the chick heart

system but not in the beef heart system, emphasize the need for extreme caution in generalizing solvent effects to other experimental conditions Similarly, their use of intact liver mitochondria to demonstrate the inhibitory effects of isooctane extraction on succinic oxidase activity cannot necessarily be applied to fractionated particles derived from mitochondria Although their particular experimental results can be explained by their proposed hypothesis that isooctane and hexane are inhibiting by conceivably forming a block between enzyme and substrate, there are equally reasonable explanations involving tocopherol which would fit in as well with the data of Nason and colleagues For example, tocopherol may be binding cytochrome b or some other respiratory chain component to cytochrome c (or one of its species) and is displaced or "unsnapped" by isooctane treatment Subsequent centrifugation or addition of tocopherol (or other active lipids) displaces the isooctane, allowing tocopherol (either endogenous or added) to fall back into its original position Alternately, restoration occurs since most of the original tocopherol still remains in the isooctane extracted enzyme Lyophilization or centrifugation may result in a concentration of the components of the enzyme making tocopherol available to the depleted enzyme sites, a hypothesis proposed earlier for the reactivation by lipid factors (Donaldson *et al*, 1958b)

The additional requirement for α tocopherol in attaining maximum restoration of succinoxidase activity by coenzyme Q in aged electron transport particles which have been centrifuged and washed so as to preclude isooctane inhibition has been also shown by Crane *et al* (1959) and Hendlin and Cook (1960) The latter workers observed a threefold stimulation in succinoxidase activity by the addition of vitamin E plus coenzyme Q over that produced by coenzyme Q alone The divergence of results and contradictory effects produced by isooctane extraction are further illustrated by the report of Igo *et al* (1959) Their highly purified DPNH oxidase preparations from beef heart mitochondria remained inactive after a 12 hour isooctane extraction, despite subsequent high speed centrifugation and successive washings which Pollard and Bieri (1959b) credit as a means of restoring activity The DPNH oxidase and DPN cytochrome c reductase activities were partially reactivated by various lipids including tocopherol and coenzyme Q, or equally well by dilution Their observations that centrifugation and subsequent reconstitution to original volume of the isooctane extracted enzyme caused a marked loss in activity which was regained by dilution are contrary to the findings of Pollard and Bieri (1959a, b) who reported that centrifugation and reconstitution of isooctane treated preparations resulted in reactivation of DPN cytochrome c reductase The results of Igo *et al* (1959) are also directly opposed to those of Nason and co workers who routinely diluted their extracted enzyme preparations

(as much as 1 200 compared to the 1 20 dilution of Igo *et al*) and never observed reactivation The fact that Igo *et al* extract their preparations for 12 hours as compared to a standard 2 *minute* extraction by Nason's group almost certainly accounts for the difference There is no question that, under the extreme extraction conditions employed by Igo *et al*, isooctane is exerting an inhibitory effect Their observations, however, that aged, extracted preparations are reactivated by tocopherol but no longer by dilution would be in keeping with the idea that a component, possibly tocopherol, had been dissociated from the enzyme Pollard and Bieri (1959c) have already reported an appreciable decrease in apparent tocopherol content of beef heart muscle during storage for 2 or more weeks at −20°C Dju *et al* (1958) had previously found a loss of tocopherol from tissues maintained at low temperature

The initial lack of specificity in the reactivation of isooctane extracted skeletal and heart muscle preparations, already indicated by Nason and Lehman (1956), Morrison *et al* (1956), Marinetti *et al* (1957b), and Donaldson *et al* (1958b) and discussed above, has been extended by other investigators Redfearn and Pumphrey (1958) observed that Tween 80, α tocopherol, and coenzyme Q restored isooctane extracted heart muscle succinate cytochrome c reductase activity, and they suggested that these compounds acted by dispersing the isooctane presumably adsorbed on the surface of the enzyme, although they indicate that isooctane may also be removing a lipid essential for activity Nason and co workers (unpublished results) had earlier observed that Tween 80 fails to restore rat skeletal and bovine heart-muscle DPN cytochrome c reductase and is in fact a potent inhibitor of the enzyme They confirmed, however, that it does reactivate the succinate cytochrome c reductase system Weber *et al* (1958a, b) found that the reactivation of isooctane extracted pig heart succinic cytochrome c reductase was brought about by compounds with isoprenoid side chains (including a number of derivatives of tocopherol, vitamin K_1, and coenzyme Q) and easily reducible quinones Vitamin A, cholesterol, stearic acid, and linoleic acid were ineffective They believe that α tocopherol and vitamin K_1 reactivate by virtue of their isoprenoid side chain rather than by their oxidation reduction properties A similar interpretation has also recently been made by Crawford and Morrison (1959) Kochen *et al* (1960) observed restoration of isooctane extracted succinate cytochrome c reductase by a branched saturated, nonpolar, long chain hydrocarbon obtained from spectral grade isooctane

Hendlin and Cook (1960), contrary to the above experiments of Weber and his colleagues, reported that isoprenoid compounds alone, other than coenzyme Q were incapable of reactivating the succinate cytochrome c reductase of isooctane extracted heart muscle particles Draper and Csal

lany (1960) concluded from their findings, in which such substances as the antioxidants santoquin and DPPD also restored the isooctane extracted heart muscle DPN cytochrome c reductase, that a long isoprenoid side chain is a necessary criterion for the regeneration of isooctane treated enzymes They felt it was remote that restoration was dependent on antioxidant activity in view of their findings that tocopherol experienced no oxidative change coincident with the reactivation of the system They suggested that inhibition is due to a purely physical mechanism such as isooctane dispersion of the enzyme These results are contrary to their earlier report (Draper and Csallany, 1958) that DPPD failed to reactivate the same system In this laboratory as originally reported (Nason and Lehman, 1956) we have failed to observe a restoration of aged isooctane treated enzyme by various antioxidants including santoquin and DPPD, whether dissolved in ethanol or dispersed in albumin, despite several attempts with different enzyme preparations Draper has since indicated (personal communication) that his observations of reactivation of the isooctane extracted enzyme with the above antioxidants occur in some cases but not in others

The earlier findings (Deul *et al*, 1958, Weber *et al* 1958a) that *d*-α tocopherol was more effective than *dl* tocopherol tended to refute the interpretation made by these workers that tocopherol reversed isooctane inhibition by removing the presumably adsorbed isooctane from the lipoprotein complex constituting the respiratory chain It was not expected that the lipid solubility of tocopherol would be affected by its stereochemical configuration A recent paper from one of the laboratories which originally reported this difference (Deul *et al*, 1958) has now observed that there is no difference between *d* and *dl* tocopherol in reactivating isooctane extracted DPN cytochrome c reductase (Berne 1960) No explanation is offered to explain these contradictory findings Although Berne also presented additional experiments illustrating the inhibitory effect of isooctane on the enzyme his observation that the addition of isooctane to the enzyme reaction mixture completely blocked DPN cytochrome c reductase is directly opposed to the results of similar experiments performed by Crawford *et al* (1959) and by Nason and Lehman (1956) Berne demonstrated an enhancement effect by tocopherol on untreated enzyme preparations identical to the observation which initiated the studies on the relationship of tocopherol to the cytochrome c reductases by Nason and Lehman (1956) Although Berne was unable to explain this enhancement by tocopherol, he suggested the possibility of an inhibitor or an increased accessibility of added cytochrome c to the enzyme system

Edwin and Green (1960) showed that the inhibition of rat liver succinoxidase by an unidentified substance from the protozoan *Tetrahymena pyriformis* was partially reversed by the addition of a number of compounds

including α tocopherol, α tocopherylquinone, and ubiquinone (but not vitamin K_1)

Donaldson *et al* (1958b), as already indicated, originally suggested that the apparent nonspecific restoration by other lipids may be due to their ability to potentiate the release of endogenous vitamin E to the active sites of the extracted enzyme The complex and ill defined effects of solvent extraction, discussed above, imply that this may be an oversimplified explanation Nevertheless, the fact that Donaldson *et al* (1958b) have been able to demonstrate that some (but not all) aged preparations of cytochrome c reductase are specifically restored by tocopherol point to a specific role for the vitamin The finding that tocopherol specificity occurs also with some aged, unextracted (as well as extracted) enzymes eliminates the possibility that the vitamin is acting by reversing or preventing inhibition by by isooctane since none was used However, the possibility has not been eliminated that with unextracted, aged preparations tocopherol is acting by reversing or preventing the inhibition by degradation products that may arise during the aging process

The recent results of Pollard and Bieri (1960) have in effect confirmed the findings of Donaldson *et al* (1958b) concerning the specificity of tocopherol in restoring aged heart muscle cytochrome c reductase preparations, and have further extended these studies They observed that DPN cytochrome c reductase, and to a lesser extent succinate cytochrome c reductase, of 1 to 10 day old digitonin treated chick heart muscle preparations were restored by the addition of tocopherol but not by tocopherol quinone, vitamin K_1, or santoquin, in agreement with the results of Donaldson *et al* (1958b) Pollard and Bieri (1960) in examining still other substances, heretofore not tested, for their ability to reactivate indicated that naphthotocopherol (formed by the reductive cyclization of vitamin K_1) and reduced vitamin K_1 were also effective The compound 5 pentadecyl resorcinol restored the succinic cytochrome c reductase of aged preparation but inhibited the DPN-cytochrome c reductase system The above active substances including tocopherol, have in common a phenolic group as well as a long nonpolar side chain In observing that some aged preparations showed a lack of proportionality between enzyme concentration and cytochrome c reductase activity (in contrast to the linear relationship obtained with freshly prepared enzyme), they concluded that an inhibitor was present On the basis of experiments whereby the dibenzoyl peroxide inhibition of cytochrome c reductase was consistently reversed by α tocopherol (but not by vitamin K_1, tocopheryl acetate, tocopherylquinone, coenzyme Q_{10}, reduced coenzyme Q_{10}, or most of the synthetic antioxidants tried), Pollard and Bieri (1960) proposed this system as a model for explaining the above effects in aged systems They attributed the above aging effect to an in

creased peroxide content of chick tissues, although they admitted that some differences exist between dibenzoyl peroxide inhibition and the inhibition observed in aged preparations They conceded that peroxides in general do not cause inhibition of enzymes of the respiratory chain and that the presence of peroxides may be fortuitous with respect to activity It therefore follows that these compounds may not necessarily be inhibitors in aged preparations The explanation for the relatively specific reactivation by tocopherol of aged muscle cytochrome c reductase, observed by Nason and his colleagues and now by Pollard and Bieri (1960), is still not clear On the one hand Nason and co workers feel that it is due to the restoration of vitamin E which as a necessary component of the enzyme system has been depleted during aging, while Pollard and Bieri on the other hand attribute it to a reversal by tocopherol of presumed peroxide inhibitors which have accumulated during the aging of the enzyme

(2) *Site of action of tocopherol* By utilizing certain dyes as terminal electron acceptors in place of cytochrome c and by use of specific inhibitors, evidence has been obtained implicating the site of tocopherol action between cytochromes b and c Inhibition by the antibiotic antimycin A, which prevents terminal electron transport between cytochromes b and c (Potter and Reif, 1952), is competitive with α tocopherol and has been shown to be partially reversed by subsequent addition of the vitamin (Nason and Lehman, 1956, Nason *et al* 1957) Vasington and Nason (manuscript in preparation) have found that of a number of lipids tested (including coenzyme Q, vitamin K_1, Tween 80, vitamin A, vitamin D, tocopherylquinone, and tocopherylhydroquinone), only tocopherol specifically prevented or delayed antimycin A inhibition of bovine heart muscle DPN cytochrome c reductase They also observed that although the demonstration of tocopherol reversal of antimycin A inhibition varied from preparation to preparation, a significant prevention of inhibition by the vitamin could be more consistently demonstrated by first exposing the enzyme to a pH ranging from 6 5 to 7 0 for a short time interval They showed that the failure of Deul *et al* (1958) to observe reversal of antimycin A inhibition by tocopherol could be attributed in part to a 5 minute preincubation of their Keilin Hartree preparation with the antibiotic and to some undefined properties of the enzyme itself Thus when Vasington and Nason preincubated a Keilin Hartree bovine heart muscle preparation for 5 minutes with antimycin A, no reversal by tocopherol occurred, whereas inhibition was significantly prevented if the antibiotic and the vitamin were added at the same time The inability of Crawford and Morrison (1959) to reverse antimycin A inhibition with tocopherol could probably be attributed to similar factors as well as to the use of relatively high levels of the antibiotic

That there is a relationship between antimycin A sensitivity and the

tocopherol enhancement effect is suggested by the correlation of these two phenomena as indicated by a literature survey in conjunction with the experimental data observed in this laboratory In all cases examined thus far, reactivation by tocopherol was associated with antimycin A inhibition, whereas a failure to demonstrate a tocopherol effect on an enzyme system was accompanied by an insensitivity of that particular organism or enzyme to the antibiotic (Nason and Vasington, 1959) This correlation is shown in Table II For example, the DPN cytochrome c reductase activities of yeast and *Neurospora*, which are inhibited by antimycin A, are restored by α tocopherol following isooctane extraction, whereas that of *Escherichia coli*,

TABLE II

CORRELATION OF ANTIMYCIN A INHIBITION (*in Vivo* AND *in Vitro*) AND TOCOPHEROL STIMULATION OF ENZYME ACTIVITY

Organism or tissue	Tocopherol stimulation	Antimycin A inhibition
Yeast (DPN cytochrome c reductase)	+	+
Escherichia coli (DPN cytochrome c reductase)	−	−
Neurospora crassa (DPN cytochrome c reductase)	+	+
TPN cytochrome c reductase (liver, muscle)	−	−
Particulate DPN cytochrome c reductase (muscle)	+	+
Acid solubilized DPN cytochrome c reductase (muscle)	−	−
Digitonin solubilized DPN cytochrome c reductase (muscle)	+	+
Microsomal DPN cytochrome c reductase (liver)	−	−

an organism which is insensitive to the antibiotic, is unaffected by either antimycin A or the vitamin This correlation together with the observed competitive inhibition between tocopherol and the antibiotic at the enzymatic level imply that tocopherol and antimycin A are acting at the same site in the terminal respiratory chain and have also led to the tempting suggestion that antimycin A may be an antimetabolite of vitamin E (Nason and Vasington, 1959)

Inhibition by 2 *n* heptyl-4 hydroxyquinoline *N* oxide which also prevents electron transport in the respiratory chain between cytochromes b and c (Lightbown *et al*, 1956) with succinate or DPNH as the electron donor, was unaffected by added tocopherol (Vasington and Nason, in preparation), as was the inhibition by Amytal, which acts between DPNH and cytochrome b (Estabrook, 1957)

Further supporting evidence indicating that the site of tocopherol action is between cytochromes b and c has been provided by experiments using 2,6-dichloroindophenol or ferricyanide as electron acceptors in place of cytochrome c In these experiments using rat skeletal and bovine heart muscle preparations neither antimycin A nor tocopherol had an effect when DPNH served as substrate (Nason and Lehman, 1956, Nason and Vasington, 1959) Moreover, isooctane extraction also had no effect on dye reduction, whereas the rate of cytochrome c reduction, which was decreased 50–90%, was restored upon addition of tocopherol

Although it has been possible to demonstrate that tocopherol acts in catalytic quantities in the enzymatic oxidation of DPNH (Nason and Lehman, 1956), there is still no evidence concerning its mechanism of action The two most likely possibilities are that it is functioning as (1) an electron carrier, undergoing oxidation reduction changes during cytochrome c reductase activity, or (2) a cementing substance, perhaps part of a lipid sheath that maintains cytochromes b and c in a suitable spatial configuration for optimal electron transport With regard to the first possibility, there is no evidence thus far of a reversible oxidation change in tocopherol during cytochrome c reductase activity (Nason and Lehman, 1956, Duel *et al*, 1958) It is conceivable, however that the quantity of tocopherol actually undergoing reversible oxidation, perhaps to a free radical or semiquinone form (Michaelis and Wollman, 1950), at the active sites of the enzyme is too small to be detected by present methods A possible role for tocopherol in oxidative phosphorylation is discussed in the next section The alternative proposal that tocopherol may be serving as a binding substance is a distinct possibility There is no evidence for or against it As to an activated form of tocopherol, little can be cited other than the observations of Morrison *et al* (1956) and Donaldson *et al* (1957), who reported an initial lag period before maximal reactivation of isooctane extracted cytochrome c reductase by added tocopherol The fact that this lag period could be overcome by a 10 minute preincubation of enzyme with tocopherol might be due not only to a conversion of tocopherol to an activated form, but alternatively to a reassociation of tocopherol with the enzyme In view of the recent evidence (Sections III,1, III,2,e,ii) correlating decreased cytochrome c reductase activity (which accompanies aging) with the accumulation of peroxidation products, a third possibility exists that tocopherol as a component of the enzyme serves to prevent by virtue of its antioxidant properties, the autooxidation of some of the lipid components comprising the respiratory chain

3 Oxidative Phosphorylation

Whether or not vitamin E participates directly in oxidative phosphorylation mechanisms is still an unsettled question The evidence to date indi

cates, however, that a vitamin E deficiency does give rise to a disturbance in phosphorylation in certain tissues There are no data to suggest whether this is due to an antioxidant effect or to a more direct role of tocopherol

The early reports that a vitamin E deficiency results in an increased oxygen uptake, which is suggestive of an uncoupling effect, led to the proposal that tocopherol is involved in oxidative phosphorylation Boyer (1943) observed a decreased formation of phosphocreatine by minces of dystrophic rat muscle, and Hummel (1948) similarly found a decreased phosphorylation of creatine by dystrophic guinea pig muscle homogenates as well as lowered ATPase activity Subsequent studies (Jacobi *et al*, 1950b, Carey and Dziewiatkowski, 1949, Weinstock *et al*, 1954, 1955), however, reported no change in ATPase activity of vitamin E deficient tissues, and in one case tocopherol actually caused a decrease (Westgren, 1950) Myers and Slater (1957), on the other hand, observed no effect of tocopherol on the ATPase activity of Keilin Hartree muscle preparation Seidel and Harper (1960) claimed a trend toward lower ATPase activity in muscle homogenates of vitamin E deficient guinea pigs A decreased phosphate transfer from creatine phosphate to hexosemonophosphate accompanied by normal glycolysis in vitamin E deficient rabbit muscle was reported by Carpenter *et al* (1957, 1958) An increased oxidation of tricarboxylic acid substrates by washed liver homogenates from vitamin E deficient rabbits with added ATP was observed by Weinstock *et al* (1955) This was decreased by higher concentrations of ATP or ADP without affecting the controls (Weinstock *et al*, 1956)

Most reports indicate a decrease of oxidative phosphorylation in cell free preparations from vitamin E deficient tissues Hummel (1948) observed a marked decrease in oxidative phosphorylation of homogenates of dystrophic guinea pig muscle, whereas Rabinovitz and Boyer (1950) found no reduction in oxidative phosphorylation of heart mitochondria from vitamin E deficient rabbits Martius (1955) reported a decreased phosphorus oxygen ratio by particles prepared from the diaphragm of vitamin E deficient dogs, although the liver mitochondria were unaffected McCay and Caputto (1956), however, found a diminished oxidative phosphorylation in liver mitochondria of vitamin E deficient rats and wondered, as did Chernick and Schwarz (1956), whether or not the effect was a secondary one, due perhaps to a nonspecific damage of the mitochondrial structure Frei (1957) recently reported a decreased ability to resynthesize ATP in liver homogenates from vitamin E deficient rats That deficiencies of other nutritional factors may have the same effect is indicated by the work of Bentley and Phillips (1951), who showed that a manganese deficiency in chicks significantly lowered phosphorylating activity of liver homogenates

Although there is essentially no evidence for the direct involvement of tocopherol in oxidative phosphorylation, the chemical structure of some of its oxidation products, especially the postulated hemiketal of Fig 2, has suggested the possibility of a hypothetical phosphorylated intermediate (Slater, 1960, Boyer, 1960), as shown in Fig 3 Model reactions involving oxidation of phosphorylated hydroquinones to give ATP have already been indicated (Harrison, 1958, Clark *et al*, 1958), Wieland and Patterman, 1958) More recently along these lines, Brodie and Davis (1958), using monoethyl menadiol monophosphate, demonstrated ATP formation and oxygen consumption with a cell free system from *Mycobacterium phlei*

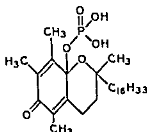


Fig 3 Hypothetical phosphorylated hemiketal of α tocopherol (see Fig 2)

IV Other Possibly Related Factors

During the last few years, two new factors, namely coenzyme Q (or ubiquinone) and factor 3 (certain selenium containing natural products), which may have some bearing on tocopherol metabolism, have received increasing attention and study No attempt will be made in this review to evaluate or summarize the literature with regard to these newly discovered substances other than to illustrate briefly what biological relationships, if any, exist between them and vitamin E

1 Coenzyme Q (or Ubiquinone)

This is the term applied to a new class of homologous quinones found in nature which have been characterized as derivatives of 2,3 dimethoxy 5 methylbenzoquinone, substituted at position 6 with a polyisoprenoid side chain Five such naturally occurring homologs, containing six to ten side chain isoprenoid units have been recognized thus far, and these have been designated as coenzyme Q_6, coenzyme Q_7, etc, respectively (see review by Green, 1959) Coenzyme Q was originally isolated by prolonged isooctane extraction of beef heart mitochondria and various electron transporting particles derived from mitochondria The resulting loss in the succinic and DPNH oxidase activities of isooctane extracted particles has been reported to be restored upon supplementation with coenzyme Q and other lipid

components which are extracted simultaneously (depending upon the length and conditions of isooctane extraction), including one or more specific phospholipid or neutral lipid fractions and α tocopherol (Crane *et al*, 1959, Hendlin and Cook, 1960) The nonspecific restoration of isooctane extracted particles by various substances, including coenzyme Q, and the implication that it also may be reversing isooctane inhibition (Redfearn and Pumphrey, 1958, Weber, *et al*, 1958a, b, Igo *et al*, 1959) have been discussed in Section III,2,e,ii The quinone has been shown to undergo a cycle of oxidation and reduction in mitochondrial particles (Hatefi *et al*, 1959), and the data suggest that coenzyme Q precedes cytochrome c as an electron carrier in the terminal respiratory chain

On the basis of present evidence, coenzyme Q and tocopherol seem to be separate and independent biological entities apparently acting at different sites in the cytochrome c reductase portion of the terminal respiratory system before cytochrome c Coenzyme Q, when administered in the diet or subcutaneously, had no effect on the muscular dystrophy of vitamin E deficient guinea pigs (Seidel and Harper, 1960) In addition to its suggested role as an electron carrier, coenzyme Q may also be involved in oxidative phosphorylation (Hatefi and Quiros Perez, 1959) The primary mechanism of action of tocopherol, possibly as a binding agent or as an electron carrier, is still unknown

Moore (1959) has pointed out that vitamin E and ubiquinone have a number of properties in common in being fat soluble, capable of undergoing reversible oxidation reduction reactions, and unstable to alkaline saponification in the presence of oxygen The two substances differ in that vitamin E is found in tissues in the reduced state mainly, whereas ubiquinone occurs chiefly in the oxidized form Within highly active cells both compounds are found principally in the mitochondria, although vitamin E is also apparently stored to a large extent in the body fat

There are conflicting reports concerning the influence of dietary vitamin E on the coenzyme Q content of rat tissues On the one hand, Moore (1959) and Morton and Phillips (1959) independently found that there was no relationship between ubiquinone content of tissues and vitamin E status thus implying that α tocopherol is neither a precursor of coenzyme Q nor a factor in any enzymatic step of its biosynthesis On the other hand, Diplock *et al* (1960) and Green *et al* (1960b) reported that a vitamin E deficiency markedly depressed ubiquinone levels in 13 tissues compared with those of stock rats and tocopherol supplemented controls They feel that "the action of tocopherol is not readily attributable to a 'protective' or 'antioxidant' effect" As further evidence they cited the results of preliminary experiments in which the use of the nonphysiological antioxidants

santoquin and DPPD, which have been reported by others to prevent certain symptoms of the vitamin E deficiency state (Section III,1), had no effect on the ubiquinone/ubichromenol levels in heart or liver

The reader is referred to an number of reviews (Nason *et al*, 1957, Nason and Vasington, 1959, Green, 1959) for a summary of information concerning other fatty components including neutral lipids and phospholipids, of the terminal respiratory system

2 Factor 3 (Active Selenium)

This is the term used to designate a biologically active selenium containing component(s) in nutrients and other biological material (Schwarz and Foltz, 1959) It was originally observed as an unidentified substance that prevented necrotic liver degeneration of rats fed a diet containing protein supplied by *Torula* yeast (Schwarz, 1951) Cystine or vitamin E will also prevent the disease The potency of cystine now appears to be due largely to contamination with minute quantities of factor 3 active selenium (Schwarz, 1960) The unclarified complexity of the biological relationship between factor 3 and vitamin E is further illustrated by the fact that either of these substances will prevent exudative diathesis in the chick (Scott *et al*, 1955), whereas factor 3 active selenium compounds do not substitute for vitamin E in preventing or alleviating muscular dystrophy of rabbits caused by a tocopherol deficiency (Draper, 1957, Hove *et al*, 1958) On the other hand, Dam and Søndergaard (1957) reported that although 70% of the chicks receiving a vitamin E-free diet developed muscular degeneration, the addition of selenium dioxide limited this degeneration to 10% of the chicks Seidel and Harper (1960) indicated that sodium selenite had no effect on muscular dystrophy of vitamin E deficient guinea pigs It was also shown that selenium did not reverse the dialuric acid hemolysis test (Gitler *et al*, 1958) and that it was ineffective in preventing resorption gestation in rats (Harris *et al*, 1958) The claim by Alterman (1959) that the inclusion of sodium selenite in a liver necrogenic diet failed to protect the incisor teeth of rats against depigmentation is opposed by the report of Irving (1959) that selenium has a significant role in curing the effects of vitamin E deficiency in the rat incisor teeth

Schwarz (1960) concluded that in essence dietary liver necrosis is the result of a simultaneous lack of vitamin E and factor 3 selenium He feels that selenium neither spares vitamin E nor substitutes for it, and suggested that their relationship to each other and the diseases with which they are associated can be classified into three groups The first group includes those diseases which are caused solely by a vitamin E deficiency and which are not influenced by factor 3 selenium, even in large excess (e g ,

resorption sterility in rats, encephalomalacia in chicks) The second group is comprised of those diseases caused only by a factor 3 selenium deficiency and which are not affected by vitamin E The third group consists of those diseases caused by a simultaneous lack of both vitamin E and factor 3 selenium (e g , dietary liver necrosis in rats, multiple necrosis in the mouse, heart and peripherol muscular dystrophy in the mink, liver necrosis and muscular dystrophy in the pig, and exudative diathesis in chicks and tur keys) Schwarz suggested that liver necrosis centered about an impairment in energy metabolism and that the synergistic partnership of vitamin E and selenium was the result of independent catalytic effects in alternate pathways of metabolism Mertz and Schwarz (1959) showed no parallelism between antioxidant potencies of a number of compounds and their capacity to influence respiratory decline in necrotic liver degeneration *in vitro* They believe that those substances which are effective in preventing the characteristic respiratory decline preceding necrotic liver degeneration are acting directly in the liver cells as catalysts in intermediary metabolism and not as antioxidants This is supported by their data that of 13 sub stances tested (including many antioxidants) characteristic respiratory de cline was prevented by addition of most compounds at catalytic dose levels A number of substances with excellent antioxidant properties were ineffec tive whether or not they are administered in the diet, intraportally, or *in vitro*

Bieri (1959) reported that dietary selenium and cystine in some indirect manner decreased the capacity of certain vitamin E deficient chick tissues to peroxidize lipides That this was not a direct action of cystine and selenium was indicated by the failure of these substances to decrease peroxidation when added to homogenates It seems improbable that se lenium is acting by sparing tocopherol in view of an earlier report (Bieri *et al* , 1958) that the rate of depletion of the vitamin from tissues was not influenced by biologically active selenium Results in the authors' labora tory demonstrated that neither selenite nor selenate could substitute in place of tocopherol in restoring cytochrome c reductase

3 *Possible Metabolic Relationship between Tocopherol and Inorganic Ions*

From the various metabolic studies of vitamin E a pattern seems to be emerging which links the role of vitamin E to the metabolism of certain inorganic ions The significant effects of Co^{++} and Mn^{++} (as well as α tocopherol) in restoring ascorbic acid synthesis of liver homogenates from vitamin E deficient rats by reactivating the gulonolactone oxidase system (see Section III, 2,e,ii) point to a possible metabolic link between these ions and vitamin E

The unexplained dietary relationship between selenium and tocopherol (see Section IV,2) in inducing certain diseases as a result of their simultaneous deficiency is an additional case in point. Green *et al.* (1960a) reported that of a number of different inorganic ions administered intraportally only Co^{++}, Mn^{++}, and $SeO_3^{=}$ showed any activity in reversing the respiratory decline in nutritional liver necrosis. Actually Mn^{++} and $SeO_3^{=}$ had only a slight effect whereas the activity of Co^{++} was proportional to the dose administered and equivalent to that produced by one half the same weight of α-tocopherol. However, the incorporation into the necrogenic diet of salts of cobalt and manganese had an opposite effect, actually causing an increase in the respiratory decline. Corwin and Schwarz (1959) had already demonstrated that Mn^{++} had a tocopherol-like effect in restoring succinate oxidation in mitochondria prepared from livers of vitamin E-deficient rats. Bunyan *et al.* (1958) had indicated earlier that of the various metal ions tested, molybdenum, osmium, and cobalt together delayed death by dietary liver necrosis beyond 137 days in three rats out of seven. In the corresponding control all 6 rats were dead after 88 days. Each of the metals separately had some protective effect. King *et al.* (1955) observed that the development of "paralysis" in mice, the result of a vitamin E-deficient diet, was influenced by mineral content of the diet. A vitamin E-deficient diet containing a complex salt mixture of trace elements failed to produce symptoms of "paralysis" in the adult mouse.

Recently Bunyan *et al.* (1960) found that the *in vitro* effects of metal ions against dialuric acid-induced hemolysis of erythrocytes fell into three groups: (1) Co^{++}, Mn^{++}, Sn^{++}, $CrO_4^{=}$, and $Cr_2O_7^{=}$ had full protective activity and were additive with the protection caused by α-tocopherol, (2) Cu^{++}, Zn^{++}, $SeO_3^{=}$, and MnO_4^{-} exerted a pronounced antagonistic effect on the action of tocopherol, and (3) numerous other ions which had no activity when tested. They suggested that the role of vitamin E in preventing hemolysis may be related to enzymatic or other type of catalytic processes in the cell and that a simple theory of combination with peroxide may not be sufficient to account for this phenomenon.

Finally Magar and co-workers (Nair *et al.*, 1956, Nair and Magar, 1958a, 1958b) in examining a metabolic interrelationship between molybdenum and vitamin E reported that the increase in alkaline phosphatase activity of rat liver and serum resulting from a high level of dietary molybdenum is counteracted by simultaneous supplements of vitamin E. In addition they observed that dietary molybdenum induced lipid depletion and necrosis in rat adrenal cortex presumably by suppressing suprarenal cortical function. The administration of the vitamin reversed this effect as well as an effect of molybdenum in inducing creatinuria. Tocopherol also appeared to be

responsible for a decrease in excretion of molybdenum by an *in vivo* retention in the tissues

The various papers cited above are highly indicative of a metabolic tie in between tocopherol and a number of metal ions Clarification of this relationship should throw some light on the functions of the vitamin

V Concluding Remarks

The numerous contradictory findings and claims in virtually every experimental area of vitamin E metabolism reflect the great difficulty in pinpointing the mechanism of action of tocopherol let alone its role in the living cell

While it is apparent from all that has been said above that the mechanism(s) of action of vitamin E in the living cell has not yet been clearly elucidated, evidence has accumulated which definitely points to at least two separate and important biological roles for tocopherol These are its involvement as (1) an antioxidant, and (2) a component of the cytochrome c reductase portion of the terminal respiratory chain, functioning perhaps directly as an electron carrier or indirectly as a binding agent A third possible role for the vitamin, namely in nucleic acid metabolism, seems to be making itself evident, largely as a result of the work of Dinning and his colleagues

Whether or not the primary role of vitamin E in the living organism is that of an antioxidant has been a subject for speculation and debate over the years There is little question that certain syndromes induced by a vitamin E deficiency are solely related to the antioxidant properties of tocopherol, since they can be completely eliminated by furnishing other antioxidants and factors in the diet However, the fact that certain other characteristic deficiency symptoms are specifically relieved by providing the vitamin speaks for a definite and specific role for tocopherol in the organism This function may well reside in its action as a component of cytochrome c reductase of striated muscle The evidence in favor of such a role is as follows (1) Tocopherol is present in rat skeletal and bovine heart muscle in amounts similar to other known enzymatic cofactors (2) The vitamin is also present in appreciable quantity in the most highly purified preparations of DPN cytochrome c reductase (3) Progressive removal of tocopherol from the enzyme by either aging or isooctane extraction results in a corresponding decrease in enzymatic activity (4) The enzymatic activity is specifically and completely restored by the tocopherols, although this cannot be consistently demonstrated from one preparation to another (5) A competitive relationship can be demonstrated between tocopherol and antimycin A, as well as a correlation in various

systems between antimycin A sensitivity and tocopherol enhancement In view of the growing evidence that a vitamin E deficiency is correlated with the accumulation of presumed lipid peroxidation products, the alternate possibility presents itself that tocopherol as a component of cytochrome c reductase serves in a protective role tending to prevent the peroxidation of the other lipid components of the respiratory chain This might also apply to its action in maintaining and restoring the microsomal gulonolactone oxidase of ascorbic acid biosynthesis The emerging pattern however that tocopherol metabolism is somehow also related to the metabolism of certain inorganic ions makes the picture even more complex

The apparent involvement of vitamin E in nucleic acid metabolism also reflects an additional specific function of tocopherol in biological systems

Methodology still proves to be a serious stumbling block in studies of vitamin E metabolism For example, the procedures employed for tocopherol assay are still in need of considerable improvement and critical study, as illustrated by the case history of the reduction method (employing HCl ascorbic acid or SnCl) for the presumed tocopherylquinone This is also emphasized by the present inadequacy in quantitatively accounting for the oxidation and degradation of tocopherol during its extraction and determination The extraction procedure itself is also in need of re evaluation While there is no doubt that isooctane and heptane are potent inhibitors of enzyme activity when used under particular conditions, there is considerable danger in extrapolating these effects to other conditions (see Section III, 2, e ii)

Finally, the recent claim by Pollard and Bieri that normal chicks can be raised on tocopherol free diets and that their tissues contain no detectable vitamin E would place in question the status of tocopherol as a vitamin by present standards Whether or not these particular results reflect the inadequacy of our present assay procedures for tocopherol must await further experimentation

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Ascorbic Acid and Collagen Fiber Formation

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I Introduction

Despite the fact that the deficiency state known as scurvy has been recognized since antiquity, that it has been classically described by Walter (1746) as involving the healing of wounds as well as the maintenance of wounds that have already healed, it was not until 1919 that Aschoff and Koch, and then Hojer in 1924, carried out detailed histological studies, which were soon followed by those of Wolbach and Howe (1926), that related the action of the accessory factor (vitamin C) to proper connective tissue, and particularly collagen, formation

The early quantitative work directed toward confirming a relationship between ascorbic acid and the production of collagen was based primarily on the measurement of the tensile strength of healing wounds in normal and scorbutic animals Since it is unequivocal that the failure of wounds to heal normally, with resulting poor tensile strength, is due primarily to

the failure to produce collagen, these experiments on the relationship of ascorbic acid to wound healing reflect the role of ascorbic acid in collagen formation The work of Lanham and Ingalls (1937) followed by that of Taffel and Harvey (1938), *Hunt* (1941), and Hartzell and Stone (1942) clearly demonstrated a direct relationship between the ascorbic acid intake and the tensile strength of healing abdominal and gastric wounds Hunt (1941), Bourne (1942a), and Danielli *et al* (1945) showed that the vitamin C deficient guinea pig appeared to convert precollagen to mature collagen much more slowly than did the normal animal Later Bourne (1944) demonstrated that in young guinea pigs the tensile strength of healing skin wounds paralleled both the plasma level of vitamin C and the vitamin intake Correlated with the decreased tensile strength of the wounds of animals on low vitamin intake was the appearance of larger amounts of *precollagenous reticular fibers* A number of investigations based on histological examinations or tensile strength studies have established beyond question the validity of the relationship between ascorbic acid and collagen formation

This relationship has been further substantiated on the basis of the quantitative chemical determination of collagen in newly formed tissue either by its isolation as gelatin or, more commonly, by determining the hydroxyproline content of hot aqueous extracts (gelatin) from the tissue In addition to regenerating skin wounds, several model systems have been employed for such studies among which are the fibrous granuloma that results from the subcutaneous injection of carrageenin studied by Robertson (1952a), D S Jackson (1957), and Slack (1957), the cellophane wrapped kidney used by Robertson (1952a), and the polyvinyl sponge (Ivalon) implant suggested by Grindlay and Waugh (1951) and used by Boucek and Noble (1955), Edwards and Dunphy (1957), and Gould (1958)

Gould and Woessner (1957) studied the relationship between ascorbic acid and collagen formation quantitatively in regenerating skin and demonstrated that practically no hydroxyproline synthesis occurred in wounds made in ascorbic acid depleted animals if they were maintained on the scorbutigenic diet When ascorbic acid was administered to animals kept on such a diet for several days, collagen formation began within 36–48 hours If animals were wounded without prior depletion, the tissues apparently contained sufficient ascorbic acid to insure almost normal hydroxyproline formation even though no ascorbic acid was given subsequent to wounding If guinea pigs were wounded after varying periods of ascorbic depletion the collagen synthesis paralleled the metabolic decay curve for ascorbic acid Almost the same relationships were found by Gould (1958) to exist in the polyvinyl sponge model The carrageenin granuloma forms little collagen in animals deprived of ascorbic acid but, unlike collagen of normal fibrous

tissue of regenerated wounds and of fibrous tissue accumulated in poly vinyl sponge implants, collagen formed in these granulomas is rather quickly resorbed even if ascorbic acid is administered

In spite of the obvious relationship between ascorbic acid and collagen fiber formation, little is known concerning the exact mechanism by which ascorbic acid acts Both the cellular physiology and the biosynthetic mechanisms still await clarification

II Early Theories

Two early theories have been the basis for much of the work that has attempted to elucidate the mechanism of the interaction between ascorbic acid and collagen fiber formation Aschoff and Koch (1919) suggested that the defect in scurvy involves the inability to produce extracellular sub stance Later Wolbach and Howe (1926) suggested that the secretion of intercellular ground substance and collagenous precursor materials proceeds quite normally but that some factor is lacking which normally causes gelling and fibrillation of a precursor in the extracellular material and that this factor is not involved in the formation of fibroblasts They found from histological examination of scorbutic teeth and bones an apparent accumulation of a fluid substance, presumably secreted by the odonto blasts and osteoblasts, which remained fluid owing to the absence of a gelling factor If vitamin C was administered gelation occurred with such rapidity that they believed there was not sufficient time for new formation of collagen and suggested ' that the failure of cells to produce intercellular substances in scorbutus is due to the absence of an agent common to all supporting tissues which is responsible for setting or gelling of a liquid product " Soon after the administration of vitamin C to depleted animals an apparently homogeneous amorphous substance which stained blue with Mallory's connective tissue stain formed around the cells This was fol lowed rapidly by the formation of reticulin fibers embedded in the amor phous material which Wolbach (1933) believed to be amorphous collagen

Opposed to this view that precursor formation is normal but that some fibrillating factor is absent was the suggestion by Fish and Harris (1934) that the failure in healing is due to a disturbance of fibroblast metabolism with a consequent failure in collagen fiber formation Ham and Elliot (1938) supported this view and concluded that the primary defect in the scorbutic animal is an impairment of cellular secretion Hojer s (1924) classic work also suggested that the defect involved in scurvy was associated with im paired function of the connective tissue cell

The modern work on collagen fiber formation begins essentially with the experiments of Stearns (1940a, b) who, using the rabbit ear chamber method presented evidence that collagen fiber formation unquestionably required

the presence of fibroblasts Porter and Vanamee (1949) and Wyckoff (1952) later showed that fibroblasts in tissue culture produced collagen fibers with characteristic 640 Å spacing The chemical identification of collagen formed in fibroblast cultures, based on the accumulation of hydroxyproline, was made by Gerarde and Jones (1953)

III Influence of Ascorbic Acid on Collagen forming Cells

The fibroblast, like the osteoblast and odontoblast, is profoundly influenced by ascorbic acid deficiency Follis (1951) has characterized such deficiency by a failure of fibroblasts, osteoblasts, and odontoblasts to promote the deposition of their respective fibrous proteins collagen, osteoid, and dentine It has been suggested that the cytochemical properties of fibroblasts are identical with those of osteoblasts except for greater phosphatase in the latter In all three types the differentiated cells appear to revert to an immature cell type in ascorbic acid deficiency

MacLean *et al* (1939) could observe no histological changes in scurvy other than in the bones and in the teeth They suggested that the effect of the vitamin is related to the maintenance and function of the osteoblasts and odontoblasts and that its primary function is in the differentiation of mesenchymal cells to osteoblasts or in the stimulation of osteoblasts to produce bone matrix Gould and Shwachman (1942) and Bourne (1943) support this view Pritchard (1956) points out that in ascorbic acid deficiency there is a regression of osteoblastic to fibroblastic forms with a cessation of bone forming power, a loss of cytoplasmic basophilia, glycogen, and phosphatase When restored to ascorbic acid there is a reversion to the typical osteoblastic form and function Thorell and Wilton (1945) have shown that differentiated odontoblasts revert to an immature cell type in ascorbic acid deficiency

Meyer (1928), who studied the fibroblast in scurvy by histological methods, observed a depletion of cytoplasm, destruction of cell membranes, and vacuolization These observations were confirmed by Wolbach (1933), Persson (1953), and Penney and Balfour (1949) It is noteworthy that these changes were most pronounced if the animals had been on a scorbutigenic diet for several days before wounding If the wounds were made soon after the withdrawal of the vitamin, the cells were less deranged in appearance Gould and Woessner (1957) found an analogous situation in a quantitative study of collagen formation in regenerating guinea pig skin and were able to demonstrate that the difference was due to the fact that on the one hand the animals had been largely depleted of available ascorbic acid stores whereas on the other there probably was still sufficient ascorbic acid available to maintain the biosynthetic system to a degree Wasserman (1958) in an electron microscope study of the scorbutic fibroblast found

an enlargement of the interlamellar spaces of the cytoplasm and the appearance of wide vesicles containing a precipitate There were no fibrils present in the vicinity of such fibroblasts

Studies of wound healing indicate that there is no apparent decrease in the proliferation of fibroblasts in scurvy Williams (1959) found that the fibroblasts were immature but that their proliferative power was unimpaired Barber and Nothacker (1952) actually found increased numbers of fibroblasts in scorbutic wounds Bunting and White (1950) and Persson (1953) reported that fibroblast numbers are essentially normal in the scorbutic wound

One of the major bases for the conclusion by Wolbach and Howe (1926) that ascorbic acid acts essentially by bringing about the setting or fibrillation of a preformed gel was the promptness with which the reaction occurred after the administration of the vitamin to scorbutic animals Gould (1959) has shown, however that after scorbutic animals are restored to an ascorbic acid supplemented diet, there is relatively little collagen formed during the first 24–36 hours and that the very rapid biosynthesis commences at about 36–48 hours Gould and Shwachman (1942) found that the reversion to normal osteoblastic activity as measured by a return to normal phosphatase production, after ascorbic acid administration to scorbutic guinea pigs occurs within 24–48 hours It is reasonable to believe that other biochemical properties of the fibroblast which might be altered in scurvy are as promptly responsive to the action of ascorbic acid

IV Collagen General Characteristics

Collagen, which constitutes about 25–30% of the total protein of the mammalian body is characterized by a unique imino acid composition in which about two thirds of all the amino acid residues are made up of glycine, proline, and hydroxyproline In addition to the high concentration (approximately 14%) of hydroxyproline collagen contains a small amount (about 1%) of hydroxylysine Apart from a small amount of hydroxyproline (1 6%) found in elastin the hydroxyproline and hydroxylysine of animal tissue have been shown to be almost entirely associated with collagen and have been used as a measure of the latter The amino acid composition is also characterized by very low contents of the aromatic amino acids tryptophan and sulfur amino acids Collagen from a variety of sources when examined with the electron microscope, shows a characteristic 640 Å periodicity with a number of interband periods Naturally occurring fibrous collagen is essentially insoluble in aqueous solvents but suitable heating in water will convert it to soluble gelatin

It is interesting that the collagens of certain invertebrates, such as those found in the cuticles of *Ascaris* and *Lumbricus* show interesting variations

in amino acid composition from that found in vertebrate collagen Watson (1958) and Watson and Silvester (1958) have shown that neither of these collagens contains hydroxylysine The glycine content is typically high in each case, however, in *Lumbricus* cuticle the proline content is extremely low whereas the hydroxyproline is high, and in *Ascaris* the reverse is the case *Ascaris* cuticle collagen contains about 30% proline, this is equal to the percentage of total pyrrolidine residues in vertebrate collagen They show no low angle X-ray diffraction pattern, nor do the fibrils have the typical 640 Å banding when examined by electron microscropy

1 Insoluble Collagen

That insoluble collagen cannot be readily purified makes impossible many studies dependent upon the isolation of a pure material Two methods are commonly used to obtain it in a "relatively pure" form (1) the removal of other proteins by extraction or (2) the conversion of collagen to gelatin by autoclaving with subsequent purification of the gelatin It is quite obvious that neither of these methods is rigorous and no data are available to indicate whether what is called collagen is actually a spectrum of different sized particles of comparable amino acid composition, a relatively homogenous population of molecules, or a mixture of different proteins

2 Soluble Collagen

The possibility that there might be smaller units of collagen that could pass out through the cell and that precursor forms of the insoluble fibers might be more readily soluble led Orekhovitch *et al* (1948a, b) to fractionate skin proteins with mildly acidic citrate buffers, in contradistinction to the strongly acid solutions used by earlier workers They were able to remove a fraction which they called "procollagen" Further studies using labeled glycine-1-C^{14} suggested that this fraction is an early form of collagen Harkness and Neuberger (1952) repeated this work but concluded that only a portion of skin collagen could be derived from "procollagen"

Highberger *et al* (1951) were able to extract a soluble component with mildly alkaline buffer solutions which they call "tropocollagen" and which is capable of being reconstructed *in vitro* into normal fibers Gross *et al* (1954, 1955) and Harkness *et al* (1953, 1954) by a similar procedure isolated an "alkali soluble collagen" which showed a very high rate of glycine incorporation and appeared to represent a much earlier stage in collagen formation than "tropocollagen" Gross *et al* (1955) have isolated a soluble fraction using 0 45 *M* NaCl as a solvent, and Jackson and Fessler (1955) have isolated a fraction using 0 2 *M* NaCl When these soluble fractions are kept at 37°C they form gels consisting of networks of fine fibrils having the typical 640 Å periodicity of native collagen Gross (1956) has re

constituted fibrils from solution under a variety of conditions and has demonstrated a variety of morphological characteristics.

It is suggested that the tropocollagen particle, found to have a length of approximately 3000 Å with a diameter of 14 Å and molecular weight equal to 340,000 is the basic building block for the formation of collagen fibers *in vivo*. The amino acid composition of neutral salt extracted collagen, citrate-extracted collagen, and insoluble collagen are essentially the same. *These substances must be considered to be morphological precursors rather than biochemical precursors of collagen.*

D. S. Jackson (1958), D. S. Jackson and Bentley (1960), and Green and Lowther (1959) suggest that there are no clear cut fractions and that neutral salt extracted collagen consists of a continuous spectrum of aggregates of collagen molecules of varying size and strength of cross linkage and that the collagen extracted with isotonic saline solution appears to be the fraction most closely associated with the cells.

Orekhovitch (1955) has shown that not only was much less procollagen found in the skin of old than of young animals, but only about one half as much was found in the skin of scorbutic animals as compared with normal. Gross (1959) has found that the skin of severely scorbutic animals contained no detectable salt extractable collagen. Similar results have been obtained by Gould (1958) in a study of collagen formation in polyvinyl plastic sponges implanted in normal and scorbutic guinea pigs. It appears that the defect in collagen formation associated with ascorbic acid deficiency involves not merely the inability to fibrillate a tropocollagen precursor, but rather one involving the biosynthesis of the soluble precursor itself.

V. Site of Collagen Fiber Formation

Considerable confusion exists as to whether collagen fiber formation occurs entirely intra- or extracellularly or both ways. The early literature has been reviewed by Klemperer (1955). Porter and his school (Porter, 1952, 1953, 1956; Porter and Vanamee, 1949; Porter and Pappas, 1959) present much evidence from electron microscope studies that fiber formation occurs extracellularly and that the unit fibrils of collagen form in close association with the cell surface. They claim never to have observed fibrils within the cell. When unit fibrils form in bundles it appears as though templates, possibly coinciding with stress fibers within the cell cortex, influence the polymerization of the fibrils out of material at the cell surface. The fibrils are shed into the intercellular spaces and grow by accretion of materials from the general milieu. These workers have been unable to repeat the experiments of Doljanski and Roulet (1933), who reported that fibers could be formed without benefit of contact with the cell. In another set of ex-

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periments designed to prove that fibers form extracellularly, Robbins *et al* (1955) showed that fibers failed to form in tissue cultures of fibroblasts grown in the presence of anticollagen serum Instead, amorphous masses of material formed, having the functional properties of collagen but without the periodicity characteristic of collagen It is suggested that a nonfibrous precursor of collagen is secreted into the extracellular space and forms an antigen antibody complex preventing normal fiber formation

As early as 1903 Mallory described slender fibrils inside the fibroblast cytoplasm and it was thought that collagen fibers were extensions of these fibrils Bang and Gey (1948) also identified by electron microscopy fibers within the fibroblast which ran parallel to the cell edge S F Jackson (1955, 1957), in a series of investigations of living and fixed tendon and bone of avian embryo, has concluded that the morphological picture of sections of fibrils cut transversely to the bundle axis indicates that fibrils are formed both intra and extracellularly (1) Groups of fibrils have been found well within the cytoplasmic areas and are ultimately extruded from the cell by some yet unknown method (2) With increase in age, fibrils form outside the cell but in close association with the cell surface According to her theory, the transformation of the soluble molecules into their fibrous form may be due to environmental change or to the action of organic sub stances or of enzymes, or both, present in the interstitial fluid This con version may occur as a direct result of cellular secretions into the inter cellular regions and subsequent reaction with the fluid, or possibly the surrounding fluid may enter the cell by pinocytosis and the cell secretion would then interact within the cytoplasm to produce intracellular fibrils It is also possible that small groups of newly formed collagen fibrils may be introduced into the cell by phagocytosis and appear to have an intra cellular origin

S F Jackson (1957) has demonstrated many cytoplasmic granules in the collagen forming cells when intercellular material is about to be or is being deposited Qualitative cytochemistry has shown the granules to con tain protein, hyaluronic acid, phosphatase, and cytochrome oxidase It has been postulated that they have fibrogenic properties and are concerned in synthetic processes associated with the formation of intercellular materials The presence of phosphatase in these cytoplasmic fibrogenic particles is noteworthy and may account for the phosphatase associated with certain newly formed collagen fibers

Green and Lowther (1959) claim to have evidence that the microsome fraction is the intracellular source of neutral salt soluble collagen They found that bound hydroxyproline isolated from the microsomal fraction after incubation of carrageenin granuloma slices with labeled proline had nearly four times the specific activity of neutral salt soluble collagen hy

droxyproline The microsomal hydroxyproline containing material was extractable with 0 14 *M* sodium chloride and coprecipitated with neutral salt soluble collagen extracted from fibrous tissue this suggests that these are probably identical and that microsomal collagen is the first collagen to be formed

VI Phosphatase and Collagen Formation

Fell and Danielli (1943), using histochemical methods, found that in regenerating connective tissue of healing skin wounds alkaline phosphatase was present and that the maximum activity of the enzyme coincided with the formation of new collagen fibers The fact that there was little phosphatase present before fiber formation and little in the later stages of regeneration, except for that associated with fibroblasts and capillaries, led them to suggest that "phosphatase is connected directly or indirectly with the metabolic processes more intimately concerned with the laying down of collagen" Danielli *et al* (1945) extended the work to a study of the alkaline phosphatase activity of healing wounds in normal and scorbutic guinea pigs No significant fiber formation occurs in the scorbutic animals and there is no phosphatase activity In partial scurvy there appears to be a parallelism between the degree of fiber formation and of phosphatase activity

The implied relationship between ascorbic acid metabolism and phosphatase rests in part on the observations of Scoz *et al* (1937) and Todhunter and Brewer (1940), who showed that the serum alkaline phosphatase in scurvy is low Shwachman and Gould (1942) and Gould and Shwachman (1942) in further chemical investigations of the serum phosphatase in scurvy showed that the serum enzyme level is a reflection of osteoblastic activity Upon the withdrawal of ascorbic acid and with the earliest onset of the scorbutic process, morphological changes occur in the osteoblasts which are accompanied by changes in their ability to produce phosphatase When ascorbic acid is administered there is a prompt return to normal morphology as well as to normal phosphatase production It was clearly demonstrated that the function of ascorbic acid was not to activate the phosphatase directly Bourne (1943) was able to confirm by histochemical methods the change in phosphatase activity in bone wounds in normal and scorbutic guinea pigs As might be anticipated, since the osteoblasts secrete such large amounts of phosphatase the fibers of regenerating bone were found to stain heavily for this enzyme Follis (1950) observed that the *Gerustmark* formed during recovery from scurvy also shows phosphatase activity

The question remains as to whether collagen fiber formation and phosphatase activity are causally related Marchant (1949) in a study of alka

periments designed to prove that fibers form extracellularly, Robbins *et al* (1955) showed that fibers failed to form in tissue cultures of fibroblasts grown in the presence of anticollagen serum Instead, amorphous masses of material formed, having the functional properties of collagen but without the periodicity characteristic of collagen It is suggested that a nonfibrous precursor of collagen is secreted into the extracellular space and forms an antigen antibody complex preventing normal fiber formation

As early as 1903 Mallory described slender fibrils inside the fibroblast cytoplasm and it was thought that collagen fibers were extensions of these fibrils Bang and Gey (1948) also identified by electron microscopy fibers within the fibroblast which ran parallel to the cell edge S F Jackson (1955, 1957), in a series of investigations of living and fixed tendon and bone of avian embryo, has concluded that the morphological picture of sections of fibrils cut transversely to the bundle axis indicates that fibrils are formed both intra and extracellularly (1) Groups of fibrils have been found well within the cytoplasmic areas and are ultimately extruded from the cell by some yet unknown method (2) With increase in age, fibrils form outside the cell but in close association with the cell surface According to her theory, the transformation of the soluble molecules into their fibrous form may be due to environmental change or to the action of organic sub stances or of enzymes, or both, present in the interstitial fluid This con version may occur as a direct result of cellular secretions into the inter cellular regions and subsequent reaction with the fluid, or possibly the surrounding fluid may enter the cell by pinocytosis and the cell secretion would then interact within the cytoplasm to produce intracellular fibrils It is also possible that small groups of newly formed collagen fibrils may be introduced into the cell by phagocytosis and appear to have an intra cellular origin

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line phosphatase activity in normal and regenerating peripheral nerves in rabbits found that the enzyme activity was not particularly localized on new collagen fibers even in the scar region where collagen was forming at a rate comparable to that in healing skin wounds Bunting and White (1950) studied the occurrence of alkaline phosphatase in skin wounds of partially scorbutic and normal guinea pigs In general, healing was retarded in partial scurvy, the wounds contained immature appearing fibroblasts, were rich in reticulin and relatively poor in collagen The alkaline phosphatase of fibroblasts and intercellular material was absent or greatly reduced in wounds of the scorbutic group, but there was no correlation between the amounts of this enzyme demonstrable and the collagen present in individual animals

Robertson *et al* (1950) tested the hypothesis in an ingenious manner They wrapped cellophane around the kidneys of guinea pigs according to the technique of Page (1939) and permitted perinephritic capsules to form The capsules showed a gradual structural transition from compact collagenous tissue on the inner zone through successively less compact fibrous tissue to a fibroblast rich outer layer containing no fibers When tested by the Gomori technique at various stages of development, it was found that in no case did fibers exhibit any evidence of phosphatase activity The nuclei and cytoplasm of fibroblasts showed some apparent activity Those at the outer periphery of the capsule showed activity associated with the nuclei When these sections were studied by the Menten histochemical technique neither collagen fibers nor fibroblast nuclei were stained The authors mention other experiments in which either cellophane was implanted or dicetylphosphate was injected subcutaneously to elicit collagen formation In neither case was phosphatase associated with the newly forming fibers It is interesting too that Pirani and Levenson (1952) could show no phosphatase activity associated with newly forming fibers in the healing of linear laparatomy wounds Brown (1953) was unable to detect alkaline phosphatase at any stage during the formation of the byssus thread of *Mytilus* Johnson and McMinn (1958) studied fibroplasia in the cat, which has no accessory ascorbic acid requirement, and found that the fibrous tissue elements formed during wound healing in the skin and hollow viscera were devoid of phosphatase which they feel is not essential for the production of fibrous protein

The apparent relationship between high phosphatase activity and fibrogenesis, at least in skin, raises the question as to whether fibrous proteins at some stage in their formation may not adsorb phosphatase from surrounding fluids cytoplasmic or extracellular, and concentrate the enzyme so that the highly sensitive Gomori method can visualize it It is possible that in the absence of fibers the enzyme is so diffuse or is removed so rap

idly from the area that it avoids detection Gould and Gold (1951) studied the degree of affinity between newly formed fibers of healing wounds and the phosphatase associated with them and found that the bond was strong enough to withstand extensive washing, treatment with trypsin, hyaluronidase, deoxyribonuclease, or ribonuclease This suggested that the bond between phosphatase and collagen is a direct and firm one In another experiment a clear fibril free preparation of purified rat tail tendon collagen was precipitated as fibers in a dilute solution of highly purified alkaline phosphatase, and it was found that considerable enzyme was firmly bound under their conditions These observations are reminiscent of those of Hoagland *et al* (1942) who found that vaccinia bodies adsorb phosphatase firmly, and of Kalckar (1944) who was able to show that adenosinetriphosphatase (ATPase) can be adsorbed firmly on precipitated myosin

In another investigation Gold and Gould (1951) applied a less sophisticated but more direct approach to testing the possibility of a causal relationship between fibrogenesis and phosphatase activity Massive amounts of very active phosphatase were repeatedly injected into healing wounds and other wounds were similarly treated with a variety of phosphatase inhibitors Neither treatment observably influenced the rate of healing of wounds when compared to normals Other experiments indicated that there was no correlation between the rate of healing and the serum levels of phosphatase which ranged from 12 to 440 Bodansky units There was no essential difference in the intensity of the phosphatase associated with the newly formed collagen in wounds in animals with high or low serum phosphatase levels

The data that have been accumulated from several investigations using a variety of material and methods do not support unequivocally a causal relationship between phosphatase and fibrogenesis It is possible that the relationship may exist in some tissues and not in others It appears not unlikely that in the metabolism of bone and teeth, where ascorbic acid does control in part the formation of phosphatase through its action on the osteoblasts and odontoblasts, there may be a close relationship to bone and dentine formation On the other hand a similar relationship with respect to fibroblastic activity and collagen formation is not so obvious

VII Possible Accumulation of Collagen Precursor in Ascorbic Acid Deficiency

Collagen uniquely contains two amino acids, hydroxyproline and hydroxylysine, which cannot be directly incorporated into protein Stetten and Schoenheimer (1943) have shown that when labeled proline is fed it is incorporated into collagen as both proline and hydroxyproline Stetten (1949) further showed that if labeled hydroxyproline was fed it was not

used to any significant extent in the biosynthesis of collagen She concluded that the hydroxyproline of collagen is derived from proline and that the conversion occurs after the proline has been incorporated into a peptide or larger molecule A similar situation has been found with respect to hydroxylysine by Van Slyke and Sinex (1958) and by Piez and Likins (1957) It is interesting that Steward *et al* (1958) studying the biosynthesis of a soluble proline hydroxyproline containing protein produced by actively growing carrot tissue cultures has observed an almost identical relationship Hydroxyproline is not directly incorporated into the protein but appears to be formed by the oxidation of proline only after the proline is bound in the protein The rate of conversion of proline to hydroxyproline is extremely rapid as compared to the rate in animal tissue Like collagen, the hydroxyproline proline containing protein is relatively inert

Several possible explanations have been put forward for the apparent inability of the animal to utilize either hydroxyproline or hydroxylysine directly (1) Hydroxyproline may not reach the intracellular sites of fibrogenesis Green and Lowther (1959) have presented evidence that this is not likely (2) It is possible that a precursor protein is formed which contains a high percentage of proline with little or no hydroxyproline In subsequent biosynthetic steps the proline of such a possible precursor is partially oxidized to hydroxyproline Robertson and Schwartz (1953) showed from a study of collagen biosynthesis in carrageenin granulomas in scorbutic guinea pigs that the administration of ascorbic acid leads to a much more rapid deposition of collagen than that encountered in natural repair The scorbutic carrageenin granuloma contains protein which, like collagen, is extractable by autoclaving, soluble in hot trichloroacetic acid, and precipitable by tannic acid, however, it contains little hydroxyproline The amount of the protein present was found to be almost equal to the amount of collagen in a normal granuloma and was rapidly replaced by collagen when ascorbic acid was administered It was suggested that in ascorbic acid deficiency there is an accumulation of large amounts of a collagen precursor However, when this protein was analyzed for its amino acid content it contained no excessively high concentration of proline or glycine and had a markedly different amino acid pattern from collagen

Gould and Woessner (1957) in a study of collagen biosynthesis in healing skin wounds in normal and scorbutic guinea pigs found that ascorbic acid depleted animals restored to ascorbic acid at the time of wounding or animals on a normal diet produce the bulk of hydroxyproline between the sixth and eighth days after wounding whereas similar animals maintained on a scorbutigenic diet produce no hydroxyproline However upon the administration of ascorbic acid 10–12 days after wounding, the animals maintained on the scorbutigenic diet produce relatively large amounts of

collagen within 48 hours This rapid formation of hydroxyproline suggested that a precursor along the lines proposed by Robertson and Schwartz might be involved A study of the amino acid changes in scorbutic granulation tissue and in implanted sponges after the administration of ascorbic acid indicated a sharp drop in the noncollagenous proline content with a concomitant increase in hydroxyproline This led to the postulation of a presumptive proline rich collagen precursor which accumulates upon ascorbic acid deprivation After the administration of the vitamin, this protein pool might be converted to a more immediate collagen precursor, perhaps tropocollagen rich in hydroxyproline It was postulated that the biochemical defect in scurvy is the inability to carry out the hydroxylation of proline to hydroxyproline, and presumably of lysine to hydroxylysine

Robertson *et al* (1959) reinvestigated this problem and isolated proline and hydroxyproline from carrageenin granulomas of normal and of scorbutic guinea pigs recovering from ascorbic acid deficiency after the administration of labeled proline Their results suggest that most of the collagen is completely synthesized during recovery and does not arise from a preformed proline rich precursor They found that the specific activity of the hydroxyproline in the recovery granuloma is more than 100% of that in the normal Moreover, they, as well as Green and Lowther (1959), found that the ratio of the specific activity of hydroxyproline as compared to that of proline was greater than 1 A corresponding relationship has been observed with respect to hydroxylysine incorporation by Van Slyke and Sinex (1958)

This would imply that the newly formed collagen eventually attains a hydroxyamino acid to unhydroxylated parent amino acid ratio greater than that in the pre existing collagen Van Slyke and Sinex (1958) have offered two possible explanations from the results of their studies on hydroxylysine formation after the administration of C^{14} lysine to young rats The first and they feel less probable is that collagen is a mixture of young collagen molecules in which the hydroxylysine to lysine ratio is 0 7 of the ratio in the total collagen and older collagen molecules in which the ratio is greater than that in total collagen As young collagen matures hydroxylation continues The constant content of lysine and hydroxylysine is maintained, they suggest, by synthesis, maturation, and breakdown of collagen molecules

The second hypothesis assumes that rat skin collagen consists of two proteins or groups of proteins one a long lived true collagen and the other a short lived metabolically active "x protein " The "x protein" incorporates lysine, but cannot have its lysine hydroxylated and contains no hydroxylysine If as a metabolically active protein it is assumed that it incorporates C^{14} lysine more rapidly than true collagen does, the specific activity of ly

sine in this protein will at first be greater than in true collagen Owing to the rapid turnover of the "x protein," the specific activity of the lysine in it would gradually fall to below the specific activity of the lysine in the true collagen and the ratio of specific activity of hydroxylysine to that of lysine in collagen "x protein" mixture would rise above 1 Robertson *et al* (1959) believe that these ratios of specific activities of hydroxyproline to proline greater than 1 suggests that the imino acids enter the collagen molecule as separate entities and that there probably exists an "active hydroxyproline" pool derived but distinct from the "active proline" pool In ascorbic acid deficiency the conversion of the "active proline" to "active hydroxyproline" is diminished

Gould *et al* (1960) have attempted to isolate proline rich precursor material from granulation tissue of animals and from subcutaneously implanted polyvinyl sponges in guinea pigs deprived of ascorbic acid Analysis of a variety of extracts yielded no fraction that was unusually rich in proline and glycine Analysis of various extracts obtained by starch zone electrophoresis also failed to yield positive evidence of such a precursor

An experiment analogous to that performed by Robertson *et al* (1959), who used carrageenin granuloma, was carried out by Gould *et al* (1960) using implanted polyvinyl sponges Guinea pigs deprived of ascorbic acid were given proline C^{14} during the days just after wounding and maintained on the scorbutigenic diet for 12 days Another group of animals were maintained on the scorbutigenic diet for 12 days after wounding, at which time they recieved both proline C^{14} and ascorbic acid The results of analyses of collagen proline and hydroxyproline from both groups gave no evidence of accumulation of labeled hydroxyproline in those animals treated with labeled proline during the period between wounding and ascorbic acid administration Actually, large amounts of proline were converted to hydroxyproline in the animals receiving both proline and ascorbic acid simultaneously The specific activity of both proline and hydroxyproline in the newly formed collagen declined rapidly in the group that had received proline C^{14} prior to the administration of ascorbic acid, whereas it remained substantially constant in the group that received proline C^{14} together with ascorbic acid In agreement with the findings of Robertson *et al* (1959) and Green and Lowther (1959), the ratio of the specific activity of hydroxyproline as compared to that of proline was greater than 1 in all animals

Gross (1959) in a study of the effect of ascorbic acid deficiency on the neutral salt extractable collagen of guinea pig skin claims to have found no excess of noncollagenous bound proline in scorbutic skin as compared with normal, nor did fractionation of these neutral salt extracts reveal the presence of significant amounts of a soluble component containing unusual proportions of glycine and proline relative to hydroxyproline One of the

fractions however, contained ten times as much proline and six times as much glycine with respect to hydroxyproline as did the normal Even though this fraction constitutes only 3% of the total nondialyzable solids of the extracts from scorbutic skin and 1% of those from normal skin, it is not inconceivable that it might be of interest

In spite of the fact that hydroxyproline is derived from proline and that the conversion appears to occur after the incorporation of the proline in some activated form, peptide or larger molecule, only persumptive evidence has been presented for the occurrence of any such precursors

VIII Direct Interaction of Ascorbic Acid in Collagen Formation

The question whether there is a direct interaction of ascorbic acid in collagen fiber formation has been studied by two methods (1) collagen fiber formation in tissue cultures of fibroblasts from a variety of sources and (2) *in vivo* fibrogenesis

1 *Tissue Culture Studies*

Jeney and Toro (1937) studied fiber formation in 7-day chick embryo tissue cultures and concluded that high levels of ascorbic acid (1700 μg per milliliter) in the medium enhanced collagen formation They found that cultures containing supplements of both embryo extract and ascorbic acid were superior to those containing embryo extract alone Querido and Gaillard (1939) found little fiber formation when scorbutic guinea pig plasma was used in the nutrient fluid of chick osteoblast cultures However, when ascorbic acid was added to such cultures (100 μg per milliliter) fiber formation was restored They claim that when normal serum was supplemented with ascorbic acid enhanced fiber formation was observed On the other hand, Hass and McDonald (1940), who cultured guinea pig spleen fibroblasts in media containing high or low levels of ascorbic acid, could show no difference in collagen fiber formation Di Fiori and Sacerdote de Lustig (1943) and Sacerdote de Lustig (1944) cultured chick fibroblasts in normal and scorbutic guinea pig plasma and in oxygenated normal plasma In media containing scorbutic plasma fewer cells seemed to develop, but they produced proportionately the same amount of reticulum as normal cells The addition of 50 μg ascorbic acid per milliliter of culture medium affected cell growth but did not influence the amount of collagen produced per cell They concluded that the vitamin is not required for collagen formation but may indirectly determine the amount of collagen formed by its action on the growth rate of cells Woessner and Gould (1957) have shown that in none of the early investigations were the media free from ascorbic acid Practically all contained embryo extract which is rich in ascorbic acid, and where oxygenation was used to destroy residual ascorbic acid it

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was shown to be ineffective These authors, using the roller tube techn carried out quantitative studies of collagen formation by chick embr lung tissue grown in media deficient in, or completely lacking, asc acid Cell growth and collagen formation were found to proceed quite in cultures lacking ascorbic acid Ascorbic acid added to whole em extract media, dialyzed media, or a completely synthetic medium found to have no appreciable effect on cell growth or total collagen fo tion Barnett and Bourne (1942) have demonstrated histochemically many mesenchymal cells in young chick embryos contain numerous vit C granules The granules may be involved in the production of fi Although every attempt was made by Woessner and Gould to remov ascorbic acid from nonsynthetic media, and even though synthetic m could be used, it is not unequivocal that the tissue that was cultured completely depleted of ascorbic acid

Gould, Goldman, and Woessner (unpublished results), and Gould (1960) have implanted embryonic guinea pig tooth germs in the ant chamber of the eyes of guinea pigs maintained on a scorbutigenic die death of the host the histological structure of the cultured teeth was f to be relatively normal even though the aqueous was practically fr ascorbic acid during the entire period No adequate explanation is avai for the observation, but it is possible that in the absence of local str the need for ascorbic acid in collagen formation is minimized

It appears that ascorbic acid does not play a direct role in the collagen formation by cultured chick fibroblasts and does not appear t required for the extracellular formation of collagen or for normal c fibroblast metabolism Woessner and Gould (1957) propose the hypotl that ascorbic acid may not play a direct role in slow collagen synthesis as normally occurs but that it may do so in rapid collagen synthesis suc is encountered in wound healing and possibly in those situations w collagen has a rapid turnover

2 In Vivo *Studies*

The possible direct action of ascorbic acid in wound healing and coll fiber formation has been the subject of numerous investigations T have been, for the most part, clinical attempts to improve healing by application of ascorbic acid directly to wounds, or studies to show there is a relatively high concentration of ascorbic acid at sites of ti regeneration and fiber formation Saitta (1929) applied vitamin C extr to the surface of wounds of animals on normal or scorbutigenic diets claims to have observed an increase in the rate of healing Lauber (19 claims to have observed accelerated healing of wounds in mice (which h no accessory requirement for ascorbic acid) when the vitamin was applie

the wound surface Rauch (1949) also claims that the direct application of ascorbic acid to wounds accelerates healing Persson (1953) found that local treatment of skin wounds in scorbutic animals was effective when the treatment was extended over several days On the other hand Proto (1936) could show no effect of injecting vitamin C into or applying it to the surface of pigeon wounds Mann and Pullinger (1940) could find no stimulation of healing by local application of vitamin C to corneas damaged by mustard gas, and Weisinger and Guerry (1955) were also unable to demonstrate enhanced or accelerated healing of corneal burns as a result of repeated local ascorbic acid applications

Several reports indicate that there is an increase in ascorbic acid in healing wounds Lauber and Rosenfeld (1938) found that when partially vitamin C depleted animals are wounded there is an apparent mobilization of the vitamin from the tissues and organs to the wounded area Barnett and Bourne (1942) in their study of the distribution of ascorbic acid in chick embryos found that just before certain cells become chondroblastic there is an increased deposit of ascorbic acid mainly in the processes of the cells Bartlett *et al* (1940 1942) claimed that ascorbic acid accumulates around traumatized tissues and suggested that it might be a factor in the accumulation of fibroblasts and leucocytes in the area They found that the tensile strength of a scar depended directly upon the vitamin C content Reid (1948) made the interesting observation from a study of the urinary excretion of ascorbic acid after injury that there was retention of the vitamin during the period of most active healing Schilling *et al* (1953) in a study of the influence of ascorbic acid and ACTH on wound healing using the technique of implanting hollow tantalum mesh spheres or cylinders as well as studying excised wounds and linear incisions, found constantly higher levels of ascorbic acid in the tissue fluid of the healing areas than was found in the serum of the animals Zamanskii and Lopushanskii (1955) also report an increase in ascorbic acid in the wound during healing Boyd (1955) claimed that healing is to some extent dependent on the local ascorbic acid concentration This conclusion was based on studies of the healing of corneal wounds in normal and aphakic eyes The aphakic eye contains less ascorbic acid in both the cornea and aqueous humor than does the normal eye Even though the concentrations found in the aphakic eye appear to be appreciable, they may be suboptimal since healing time is appreciably lengthened and the number of relapses during healing is much greater than normal Ksab'yan (1956) has studied the ascorbic acid content of healing tissues histochemically He found that on the first and second days after making a skin wound there was practically a tripling of the ascorbic acid content as compared to normal skin (120 mg /100 gm compared to 44 mg /100 gm in normals) Histological studies indicated that

the increase occurred at the expense of ascorbic acid in the tissue surrounding the wound Ascorbic acid was found in the epithelial cells and in the early forms of fibroblasts From the third day the wound ascorbic acid level falls to within normal range then falls below it On the third day it is localized at the bottom of the wound in the newly formed granulation tissue When epithelialization sets in it is found in epithelial cells, in young fibroblasts, histiocytes endothelial capillaries, and in newly formed epithelium By the sixth day it rises to normal levels and is associated with the newly formed fibers In a recent study Abt *et al* (1960) have also reported increased concentrations of ascorbic acid at sites of healing

Gould (1958) has presented evidence for a direct specific effect for ascorbic acid in collagen biosynthesis *in vivo* Quantitative methods based on hydroxyproline synthesis as a measure of collagen formation were employed Paired polyvinyl sponge implants were made subcutaneously in previously ascorbic acid depleted guinea pigs The administration of relatively small doses of ascorbic acid directly into such implanted sponges resulted in rapid hydroxyproline synthesis with little or no synthesis in the saline injected control sponge in the same animal When ascorbic acid was administered by mouth at the same time that one of the sponges was injected, both sponges formed collagen but an additive effect was observed in the injected sponge The direct action of ascorbic acid was found to be specific for substances of known antiscorbutic activity since dihydroxy maleic acid, glucoascorbic acid, and isoascorbic acid are inactive, whereas L ascorbic acid and dehydro L ascorbic acid are active

The fact that collagen formation in polyvinyl sponge implants and in other situations of repair is unequivocally dependent on ascorbic acid and since the mechanism is directly mediated by ascorbic acid, in contra distinction to collagen formation in tissue culture which appears to be independent of ascorbic acid, the possibility of more than a single mechanism for collagen formation has been suggested Gould (in press) has tested this hypothesis and concludes that two major types of collagen formation occur (1) relatively ascorbic acid independent characteristic of normal growth and (2) an ascorbic acid dependent mechanism involved in repair

IX Role of Ascorbic Acid in the Maintenance of Collagen

Although there is little question that a relationship exists between ascorbic acid and collagen production, it is not amply clear what the relationship is between ascorbic acid and the maintenance of collagen On the basis of experiments using labeled amino acid incorporated into collagen, Neuberger *et al* (1951) and Robertson (1952b) have shown that collagen is metabolically inert compared to most other proteins However, data do

exist indicating that collagen is not completely inert but that there is a slow synthesis and degradation even in adult skin and tendon In certain tissues such as liver, bone, and periodontal membrane, the process can be relatively rapid However, Neuberger (1957) has stated that although the time for 50% loss (half life) of collagen in rat tail tendon, bone, liver, and skin differed somewhat, it was in excess of 50 days Harkness *et al* (1958) found the half life of skin collagen of mice maintained on a protein free diet to be 100 days In certain systems the disappearance of collagen can proceed at an extremely rapid rate Harkness and Moralee (1955) report the half life for the collagen in the involuting uterus as 1–2 days, in recovery from hepatic necrosis described histologically by Cameron and Karunaratne (1936) and measured chemically by Morrione (1949), it was found to be about 10 days, in the carrageenin granuloma, D S Jackson (1957) reports it to be 5–6 days in subcutaneously implanted polyvinyl sponges in the rat, Noble *et al* (1958) claim it to be about 30 days, and Gould (1960) finds that upon the withdrawal of ascorbic acid from guinea pigs bearing implanted polyvinyl sponges the half life of the newly formed collagen is also about 30 days

Some of the earliest reports of scurvy contain statements to the effect that wounds which had been healed for years would break down if the individual became scorbutic (Walter, 1748) Hunt (1941) studied the effect the withdrawal of vitamin C on healed wounds, his conclusions, based on a few animals, were that mature collagen in scars may retrogress if ascorbic acid is withdrawn from the diet for prolonged periods and that the new collagen had reverted to an argyrophilic precollagenous state very different from that found in normal control wounds Pirani and Levenson (1953) in a carefully controlled series of experiments also demonstrated that ascorbic acid is necessary for both the healing of wounds and the maintenance of the collagen which had formed They subjected guinea pigs to laparotomy and then allowed the wounds to heal for 2–6 weeks Some of the animals were then deprived of ascorbic acid while pair fed controls received ascorbic acid About 3 weeks after the withdrawal of ascorbic acid changes were observed in the wounds of many of the animals on the deficient diet In these animals the connective tissue was loose and numerous fibroblasts and immature mesenchymal cells were present There were numerous capillaries, many of which were defective, and several areas of hemorrhage The changes observed were of the same type as those that occur in healing wounds in scorbutic animals Similar histological changes have been observed in polyvinyl implants in guinea pigs which have been deprived of ascorbic acid after considerable collagen formation had taken place while the animals were fed a complete diet Elster (1950) studied the relationship of ascorbic acid to collagen maintenance in young guinea

pigs The collagen content of a number of organs and tissues of scorbutic guinea pigs and of a series of normal animals as age and weight controls was determined When animals of the same age were compared, the scorbutic animals had a lower content of collagen in several tissues, but when animals of the same weight were compared there was no difference in collagen content Similar results were obtained by Gould (unpublished results) Elster felt that once collagen is formed it is independent of further ascorbic acid nutrition except insofar as it might be destroyed and require resynthesis Robertson (1950) *also studied the collagen content of normal* and scorbutic guinea pigs and found that the collagen content of skin, liver, kidney, teeth, costochrondral junctions, lung, or spleen in acute or chronic scurvy was in no case appreciably lower than in normal guinea pigs These observations suggested that either the amount of collagen destroyed was small or that the degradation in structure was slight In a subsequent investigation Robertson (1952a) studied the effect of the withdrawal of ascorbic acid on the collagen of induced fibrous tissue formed by wounding, by subcutaneous injection of diacetyl phosphate, of carrageenin, or by wrapping a kidney in cellophane The collagen content of the healed wounds and of the new subcutaneous fibrous tissue was not lowered when the animals were made scorbutic However, under the same conditions the collagen content of the fibrous tissue about the kidney did decrease Levenson (1959) points out that the discrepancy between his findings and those of Robertson with respect to wound collagen after ascorbic acid withdrawal may be due to the fact that the measure of collagen used by Robertson is based on the hydroxyproline content and therefore would not differentiate between Van Gieson stainable collagen and reticulin Neither would it differentiate between insoluble collagen and soluble collagen Robertson concluded from his studies that ascorbic acid is not essential for the maintenance of preformed collagen and that possibly only certain recently formed collagen requires ascorbic acid for maintenance Since this constitutes only a small fraction of the total, it is difficult to demonstrate the relationship He suggested that the major portion of induced new collagen, like organ collagen does not require ascorbic acid for maintenance

Gould *et al* (1960) implanted polyvinyl sponges subcutaneously in ascorbic acid-depleted and in normal guinea pigs and then permitted new fibrous tissue to accumulate After varying periods of collagen formation the animals were placed on a scorbutigenic diet and the rate of collagen "resorption" from the sponges was determined chemically It was found that almost directly after the withdrawal of ascorbic acid there was complete disappearance of salt soluble collagen and this was followed by the slower disappearance of insoluble collagen They found that the rate of resorption was greater from sponges with newly formed collagen than from those

which had been implanted for a prolonged period, but the latter also showed considerable resorption When sponges were implanted in animals that had not been depleted there was considerably less resorption This is difficult to explain since, even though the animals had been depleted, once the sponges were implanted ascorbic acid was administered for as long as 30 days before the animals were placed on the scorbutigenic diet Histological examination, however, suggests that the difference may involve impaired blood vessel formation during the first few days of organization of the implant, during which time the animal has not been resaturated with the vitamin It is interesting that Gillman (1957) has suggested that for collagen of certain tissues, such as arteries, evidence indicated a far shorter life span or a more rapid turnover than hitherto expected, especially in the presence of either metabolic or physical injury

The mechanisms involved in collagen "resorption" are little, if at all, understood It is not even clear whether the process involves collagenolysis by specific or nonspecific enzymes or merely removal of the particulate material by phagocytosis Hunt (1941) suggested that scar collagen was converted to a precollagenous state after the withdrawal of ascorbic acid D S Jackson (1957) found that during the breakdown period of carrageenin granuloma tissue there was a rise in the soluble collagen fractions indicating that the mature or insoluble collagen fibers were being broken down prior to removal Gould (1959) however could find no accumulation of soluble collagen during resorption, but rather a disappearance of it It is possible that it is removed as rapidly as it is formed Boucek *et al* (1959) point out that tissue formed in implanted sponges in rats contains a number of proteases and peptidases, including prolidase, and prolinase and find that these enzymes reach their maximum levels after the rate of collagen accumulation has passed its peak and when degradation or turnover mechanisms become maximal Gould (1960) has studied the amino acid composition of collagen residues in sponges after resorption on the basis that there should be a change in the proline hydroxyproline glycine ratio from normal if collagenolysis is operative The results strongly suggest that this is so

X Ascorbic Acid, the Ground Substance, and Fibrogenesis

Considerable attention has been directed toward the influence of ascorbic acid on the ground substance and the possible role of the latter in collagen fiber formation The ground substance is the amorphous interfibrillary material thought of histologically as being the metachromatic staining (toluidine blue) and periodic acid Schiff (PAS) staining material and chemically as being composed largely of mucopolysaccharides such as chondroitin sulfate and hyaluronic acid Jackson (1958) has pointed out, however, that the ground substance is a complex mixture of mucopoly

saccharide, mucoprotein, protein, and low molecular weight substances The protein component makes up at least 4 % of the total skin protein

In healing wounds, shortly after fibroblastic proliferation but before fibrogenesis begins, there is an accumulation of material showing toluidine blue metachromasia which supposedly is a stain specific for mucopolysaccharide sulfuric acid esters The periodic acid Schiff stain, Hale's colloidal iron stain, and the hexosamine content parallel the metachromasia, substantiating the presence of mucopolysaccharide This accumulation continues for a few days and then declines rapidly, the decline occurring with the onset of fiber formation

Meyer (1943–1944) concluded from a study of the toluidine blue staining characteristics of articular cartilage in chronically scorbutic animals that the metachromasia was markedly reduced, this he believed was due to a reduction in chondroitin sulfate Penney and Balfour (1949) found that healing wounds of scorbutic guinea pigs failed to produce acid mucopolysaccharides, but after the administration of even small amounts of ascorbic acid a large amount of mucopolysaccharide, either chondroitin sulfate or hyaluronic acid, was promptly produced According to them, fiber formation first involves the deposition of mucopolysaccharide around the fibroblasts Gersh and Catchpole (1949) studied the ground substance of interscapular skin of scorbutic guinea pigs In addition to an increase in the size and number of fibroblasts, they claimed that in scurvy there is a depolymerization of the ground substance, they based this on the fact that they found an increased concentration of water soluble glycoprotein Pirani and Catchpole (1951) reported in scorbutic guinea pigs increases in serum glycoproteins which were reduced after ascorbic acid treatment They suggest that the increased serum polysaccharide level in scurvy results from the absorption into the circulation of depolymerized polysaccharides of the ground substance Persson (1953) also finds that the ground substance in scorbutic wounds contains disaggregated, depolymerized polysaccharide which gives a positive Schiff's reaction but is not metachromatic

Bradfield and Kodicek (1951) in a study of wound sections from normal and scorbutic guinea pigs found that wounds of normal animals stained by the periodic acid Schiff method showed a decrease of stainable material as healing progressed whereas in the scorbutic animal it increased They did not, however, find any difference in metachromatic staining of skin wounds of both normal and scorbutic animals, they interpreted this as indicating that the abnormal abundance of polysaccharide material in scurvy probably was not sulfated Scorbutic wounds appeared to be composed of thick, chaotically arranged precollagenous fibers suggesting the accumulation of a mucopolysaccharide sheath around a precollagen core The mucopolysaccharide supposedly interferes with the subsequent maturation of the collagen They claim that the mucopolysaccharide was neither water soluble

nor susceptible to either amylase or hyaluronidase Robertson and Hinds (1956), in a study of granulomatous repair tissue in normal and scorbutic guinea pigs produced by the subcutaneous injection of carrageenin, found that the collagen poor tissue accumulated under scorbutigenic conditions contained about five times as much mucopolysaccharide as the repair tissue formed under normal conditions On fractionation of the tissue, the polysaccharide that accumulated appeared to be hyaluronic acid, contrary to the claim of Bradfield and Kodicek (1951) Slack (1957 1958) supported the findings of Robertson and Hinds (1956) by demonstrating that there is in scorbutic carrageenin granulomatous tissue a greatly increased accumulation of polysaccharide which in fact is due to an increase in hyaluronic acid or compounds with very similar properties This hyaluronic acid has a composition somewhat different from that found in normal granulomas They also report that though the level of sulfated polysaccharides is somewhat lower in scorbutic animals it appears to be the same or very similar in character to that found in normal animals However, studies based on the incorporation of S^{35} into mucopolysaccharides indicate a reduction in the rate of metabolism of chondroitin sulfate in scurvy

Ludwig *et al* (1950) were able to show that ascorbic acid deficiency does not interfere with the normal production of mucopolysaccharides of connective tissue They treated groups of normal and totally scorbutic thyroidectomized guinea pigs with thyroid stimulating hormone to produce an exophthalmos that results from an accumulation of hydrophilic metachromatic staining substance in the orbital connective tissue Microscopic as well as chemical analyses for hexosamine revealed neither qualitative nor quantitative differences between the mucopolysaccharides formed in the normal and scorbutic animals

Kodicek and Loewi (1955) administered $Na_2S^{35}O_4$ to normal and scorbutic guinea pigs and studied the incorporation of S^{35} into regenerating tendon, they found it to be decreased in the scorbutic animals while the hexosamine content was normal From these results it would appear that ascorbic acid may be involved in the sulfation of mucopolysaccharides In this respect the early theory of Meyer (1946 1947) is interesting He suggested that the fibroblast first secretes hyaluronic acid, then chondroitin sulfate and a collagen precursor The mucopolysaccharides form chains with regularly spaced acidic groups These chains act as templates on which the globular precursor is denatured The mucopolysaccharides act as anionic detergents rolling out the peptide chains along the acidic groups As the fibers age, hyaluronic acid is removed enzymatically, leaving chondroitin sulfate on the fibers It should be noted, however, that considerable collagen formation has occurred before the maximum concentration of sulfated polysaccharide appears

Stein and Wolman (1958) also conclude, from a study of polysaccharides in healing guinea pig wounds, that the impaired healing in ascorbic acid deficient animals is accompanied by (or due to) diminished sulfation of the ground substance They too report the common finding that after the administration of ascorbic acid to deficient wounded animals repair progresses more rapidly than in normal animals They suggest that the rapid growth of granulation tissue probably indicates that the necessary materials for repair are present in the scorbutic wound but are not utilized

Gross *et al* (1952) were able to convert purified soluble collagen to normal fibers *in vitro* using mucoprotein, serum glycoprotein, hyaluronic acid, or chondroitin sulfate, but the effect was found to be nonspecific Similar results were obtained by Jackson and Randall (1956) and by Bychkov (1950) Gross (1956) found that merely altering the ionic strength of the solution will induce fiber formation if a solution is kept at 37°C, indicating that even though mucopolysaccharides may be involved in the fibrillation of soluble collagen they are not essential In studies of the influence of various electrolytes and nonelectrolytes on *in vitro* fibril formation, Gross and Kirk (1958) found that chondroitin sulfate, hyaluronic acid, and acid glycoprotein of serum had no effect on the rate or extent of fibril formation and that ascorbic acid actually had an inhibitory effect

Jackson (1958) in a study of implanted polyvinyl sponge tissue found that chondroitin sulfate and hyaluronic acid account for only about 5% of the hexosamine, 80% being associated with the plasma proteins present He found the hexosamine nitrogen ratio of the sponge tissue to be almost identical with that of the serum of the same animal and presents this as strong evidence against the essential character of mucopolysaccharide in the formation of collagen fibers in the healing wound

There is *no proof that mucopolysaccharides are specifically involved in* the formation of collagen They may have to do with other functions of these tissues and relatively little if anything to do with the formation of collagen fibrils However, it must be kept in mind that it is possible that although they may not play an active role in fibrogenesis *in vivo* they may play an important passive role

Another possible role for the ground substance in fibrogenesis has been suggested by Balasz and Holmgren (1950), who found that extracts of granulation tissue had maximum inhibitory action on fibroblast proliferation at about 9 days and that this control of the fibroblasts may possibly be associated with the mucopolysaccharide content of the granulation tissue Any role of ascorbic acid that would control the nature of the ground substance might then be reflected in fibroblast activity

Williams (1959) suggested that since mucopolysaccharide and protein synthesis probably have different metabolic pathways, the fact that ascorbic

acid appears to influence both suggests that it may be involved in very basic metabolic functions in the cell

XI THE ROLE OF HYALURONIDASE IN COLLAGEN FORMATION

The accumulation in scurvy of apparently depolymerized ground substance suggested by Gersh and Catchpole (1949) is in accord with the findings of several investigators who propose that the depolymerization of the ground substance is the result of hyaluronidase action, that under normal conditions ascorbic acid or its degradation products inhibits the enzyme but that in its absence the ground substance is depolymerized and collagen formation is prevented Reppert *et al* (1951) claimed that ascorbic acid inhibits hyaluronidase *in vitro* However, Patterson and Cole (1952) found that ascorbic acid itself did not inhibit the enzyme but that dehydroascorbic acid did and that the further oxidation products of dehydroascorbic acid are even more strongly inhibiting They suggested that ascorbic acid serves as a reservoir from which compounds are formed that control the hyaluronidase system On the other hand, it is possible that ascorbic acid deficiency leads to impaired tyrosine metabolism, with the resultant accumulation of quinones which have been shown to be effective hyaluronidase inhibitors by Roseman and Dorfman (1952) The accumulation of hyaluronic acid in scurvy observed by Robertson and Hinds (1956) and by Slack (1958) suggests that the absence of ascorbic acid may in fact result in decreased hyaluronidase activity, this according to Meyer's theory, would result in the inability of the scorbutic animal to remove the hyaluronic acid from immature fibrils and thus prevent their maturation

On a nonenzymatic level Robertson *et al* (1941) have shown that ascorbic acid and peroxide acting together were capable of degrading a variety of hyaluronic acid containing mucopolysaccharides *in vitro* Daubenmerkl (1951) studied the action of high concentrations of the mixture of ascorbic acid and peroxide *in vivo* in humans and rabbits and was able to observe a spreading effect in subcutaneous tissue analogous to that produced by hyaluronidase These observations are interesting in that ascorbic acid or its oxidation and degradation products may be involved in the removal of mucopolysaccharide which accumulates around newly formed collagen fibers

Several investigators (Baggi and Prodi, 1954 Telka and Kulonen, 1954 and Karsavina and Muzykant, 1958) have found that the application of hyaluronidase to wounds leads to the inhibition of scar formation On the other hand Gould *et al* (1960) have injected large amounts of hyaluronidase or hyaluronidase inhibitor into subcutaneously implanted polyvinyl sponges during fibrous tissue formation and has observed very little impairment of fiber formation in either case

It is abundantly clear that ascorbic acid has a role in the formation and the organization of the interfibrillary ground substance The role of the latter in fiber formation, which in repair is unequivocally dependent upon ascorbic acid, has yet to be elucidated

XII Possible Interaction of Adrenal Hormones and Ascorbic Acid in Collagen Formation

The extremely high concentration of ascorbic acid found in the adrenal as well as in the pituitary gland and the fact that the injection of cortisone has been found in many cases to delay wound healing have suggested a possible relationship between ascorbic acid and cortical hormones in collagen formation Symptoms of adrenocortical insufficiency have been reported by Lockwood and Hartman (1933) to resemble those of scurvy, and according to Plotz *et al* (1950) the effects of cortisone on healing mimic the effects of ascorbic acid deprivation to such a degree that they considered the possibility that cortisone produced a scurvy like condition even in the presence of adequate amounts of ascorbic acid The literature relating to the general relationship of cortical hormones to connective tissue has been reviewed among others by Taubenhaus (1953a, b), Asboe Hansen (1954) Bourne (1952, 1956), and Edwards and Dumphy (1958)

Upton and Coon (1951) could find no evidence that either ACTH or cortisone influenced the rate of growth, nutrition, hemorrhagic tendency, or repair of wounds in normal and scorbutic guinea pigs Wolbach and Maddock (1952) have presented data which quite definitely suggest that there is no direct interaction between ascorbic acid and impaired connective tissue formation resulting from cortisone administration These workers investigated a number of guinea pig tissues histologically and found that the administration of cortisone to scorbutic animals influenced neither the rate of onset or degree of severity of scurvy, nor the repair process when cortisone was given simultaneously with ascorbic acid to scorbutic animals Bourne (1952) studied the effect of cortisone on the healing of skin wounds in rabbits and found that cortisone administration resulted in partially healed wounds with considerably decreased tensile strength The administration of large amounts of sodium ascorbate simultaneously with the cortisone did not counteract the effect of the latter The cortisone treated animals still produced a partially healed wound analogous to that found in partial ascorbic acid deficiency It is clear that cortisone does not produce its effect by depriving the animals of ascorbic acid Pirani *et al* (1952) have also concluded from a study of the influence of ACTH on collagen formation in normal, scorbutic, and ascorbic acid treated guinea pigs that the action of ascorbic acid is direct, and not through the adrenal cortex Gould (unpublished results) has implanted polyvinyl sponges subcutane

ously in guinea pigs to which relatively large doses of cortisone were administered, either parenterally or by injection into one of a pair of sponges in each animal, during the period of fibroblast formation, during the subsequent period of fiber formation and throughout the entire period of fibrogenesis Ascorbic acid was given throughout Other animals were treated with cortisone during the period of recovery after having been maintained on a scorbutigenic diet No significant differences could be detected in collagen formation, as measured by hydroxyproline synthesis, as a result of cortisone treatment in any of these cases

It appears unlikely, on the basis of the evidence presented to date, that the action of cortisone on connective tissue formation is mediated through or regulated to any large extent by, ascorbic acid

XIII Summary

Despite the voluminous literature that exists concerning the biological effects of vitamin C or ascorbic acid deficiency and even in the face of an expanding body of information regarding the structure and biosynthesis of collagen, very little can be said unequivocally regarding the specific mechanisms by which ascorbic acid may control or regulate collagen biosynthesis It is clear that the collagen formed in implanted sponges, in carageenin granulomas and in wound repair involves an interaction with ascorbic acid It is possible on the other hand that there are other mechanisms or pathways, for collagen formation such as in normal growth which do not involve an interaction with ascorbic acid In the rapid formation of collagen, as demonstrated in subcutaneous polyvinyl sponge implants, the interaction has been shown to be a specific and direct one Only substances of known antiscorbutic activity are effective, and they have been shown to operate at the site of collagen formation The action of ascorbic acid does not appear to involve an interaction with the adrenal cortex which in turn would regulate collagen biosynthesis through the cortical hormones

The site of actual collagen fiber formation is still a matter of some question, but it appears that the bulk of collagen fibers is produced by the fibroblasts extracellularly but in very close proximity to the cell surface The fibrils are shed into the intercellular spaces and grow by accretion of materials from the general milieu It is however, not impossible that collagen fibers can be formed intracellularly to some extent

The intimate association of phosphatase with newly formed collagen, which occurs in some cases, may be explained on the basis that in the course of fibrillation and of fiber growth by accretion, phosphatase associated with the fibroblast is adsorbed onto the newly formed fibers The fact that fibers have been shown in many cases to form in the absence of

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It appears unlikely, on the basis of the evidence presented to date, that the action of cortisone on connective tissue formation is mediated through, or regulated to any large extent by, ascorbic acid.

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The site of actual collagen fiber formation is still a matter of some question, but it appears that the bulk of collagen fibers is produced by the fibroblasts extracellularly but in very close proximity to the cell surface. The fibrils are shed into the intercellular spaces and grow by accretion of materials from the general milieu. It is, however, not impossible that collagen fibers can be formed intracellularly to some extent.

The intimate association of phosphatase with newly formed collagen, which occurs in some cases, may be explained on the basis that in the course of fibrillation and of fiber growth by accretion, phosphatase associated with the fibroblast is adsorbed onto the newly formed fibers. The fact that fibers have been shown in many cases to form in the absence of

phosphatase and can form even if phosphatase normally associated with the fibers is inactivated suggests that there is no causal relationship between phosphatase and collagen fiber formation The action of ascorbic acid in collagen formation would therefore not depend upon its control of phosphatase activity

There is ample evidence that the withdrawal of ascorbic acid has a profound effect on the production and nature of the ground substance, yet little unequivocal evidence exists to show that impaired collagen formation occurs as a result of such alterations in the ground substance Any role for hyaluronidase in normal fiber formation is also purely presumptive

The biochemical defect in collagen formation resulting from ascorbic acid deficiency appears to be associated with an inability of the animal to hydroxylate proline to hydroxyproline and lysine to hydroxylysine However, up to the present the numerous attempts to isolate a proline rich precursor that accumulates in wounds or skin of scorbutic animals have met with no success

The role of ascorbic acid in collagen formation is little questioned, however, there is considerable disagreement concerning its role in the maintenance of collagen Data are accumulating which suggest that newly formed collagen or collagen formed under conditions of partial deficiency is quite extensively "resorbed" after ascorbic acid withdrawal by some yet unexplained mechanism of collagenolysis

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Nutritional Factors and Skin Diseases

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I. Introduction

From primitive times, man has related the gloss and sheen of the hair coat and skin of his pets and herds with good dietary practices and good health. In experimental nutrition studies, a change in the hair coat and skin of animals has often been the first and most outstanding feature of a dietary deficiency disease. Unfortunately, these skin and hair changes are often nonspecific, and rarely permit the diagnosis of an isolated nutritional

deficiency Because these skin changes have appeared with poor matologists have been much influenced by the use of special diets, and minerals in nearly every conceivable dosage level and comb an attempt to manage and treat skin diseases Since accurate di difficult under such conditions and since many skin diseases are of etiology, much of this form of therapy has been doomed to failur tended to place the role of nutrition in the therapy of skin disease pute There is a place for nutrition in dermatology as in medicine it should be kept, however, in proper perspective

Although dermatoses related to dietary deficiency states can a any part of the body, they tend to be located over areas of irr excessive stimulation Lesions are most apt to appear on the bac hands, on the front and backs of the wrists, on the elbows, over around the neck, under the breasts, over the knees and feet, ar perianal region These lesions are often bilaterally symmetrical an be separated from healthier appearing skin by a sharp line of dem They are generally accompanied by burning, itching and pai 1955a)

The skin area in the average adult is about 1 75 square meters of yet resilient, double layered structure which is the principle line o against the entrance of foreign substances and protects agains every conceivable physical and chemical stress When damaged remarkable ability to repair itself, thus reflecting its dynamic n activity Through its sweat glands and blood vessels, the skin pro principal means by which the body heat is regulated, and through endings it permits sensation of contact with the external envi perceiving heat, cold, pain, and other stimuli The skin also ref emotions, such as embarrassment, fear, anger, and anxiety, and, nately, a person's age as well We are even individualized by chara skin markings—our fingerprints and footprints

A brief review of the structure and physiology of the skin may b in understanding the changes that take place in skin diseases (l et al, 1956, Urback and Lewinn, 1946) The skin is composed principal layers (1) epidermis, (2) corium (dermis or true skin), the subcutaneous tissue

The epidermis is a thin layer averaging about 0 2 mm in thick which the greatest metabolic activity of the skin is located From side inward, the epidermis is composed of cell layers (1) stratum c (2) stratum granulosum (3) prickle cell layer, and (4) the basal ce Melanocyte cells responsible for the production of skin pigment, a in the basal cell layer The epidermis overlies the corium or der wavelike pattern The principal metabolic activity of the epiderm

production of the protein keratin, which is the chief component of the layer stratum corneum Keratin, by its toughness, elasticity, and resistance to chemical change, provides great protection It is structurally integrated into the flat, shrunken, nonnucleated dead cells of the stratum corneum The process of keratinization is, therefore, concerned with the production of keratin and the gradual elaboration and replacement of the stratum corneum from the basal cell layer of the epidermis This response is continuous and may vary with the stimuli on the epidermis

There are two kinds of skin appendages derived from the epidermis (1) glandular, i e apocrine and eccrine sweat glands, and sebaceous glands, and (2) keratinized structures, i e the hair follicles and hair and the nails

The corium or secondary protective layer supports the vascular bed and peripheral nerves of the skin and the epidermal appendages The corium is also a large potential storehouse for water, blood, and electrolytes This is well exemplified in the individual with edema

The subcutaneous tissue provides further support and is an excellent heat insulator a good shock absorber, and a storehouse of calories in the form of fat

II PROTEINS AND AMINO ACIDS

The proteins of plasma, especially albumin, exert an effective osmotic influence in preventing the loss of water from the vascular system Any clinical circumstance in which the plasma level of albumin falls below 2 5 gm per 100 ml often leads to the development of edema Edema due to protein deficiency is frequently a by product of war or famine, and terms like ' war edema and "hunger edema" have appeared As protein deficiency develops, a decreased ability to make antibodies (Cannon *et al*, 1943) may occur

Long exposure to experimental diets low in protein results in extensive skin changes These changes are characterized by dryness, scaliness, in elasticity, and a gray pallid appearance suggestive of old age Also noticed, is a blotchy, dirty, brownish pigmentation which may appear anywhere on the body but most often on the face (Keys 1948)

While it is attractive to relate these skin alterations noted in protein deficiency to specific essential amino acid deficiencies, this has been most difficult to establish Certain metabolic blocks involving amino acid metabolism have been reported In phenylpyruvic oligophrenia, marked skin changes occur characterized by eczematous eruptions and evidence of in adequate melanin formation (Jervis, 1937) In this condition, a genetically controlled metabolic block due to a nearly complete absence of phenyl alanine hydroxylase limits the conversion of the amino acid phenylalanine to tyrosine, and is accompanied by an increased urinary excretion of phenyl

pyruvic acid (Moldave and Meister, 1957) Abnormal amounts of indole derivatives are also excreted in phenylketonuria (Armstrong *et al*, 1955)

Since keratin, the chief protein of skin and hair, has substantial amounts of sulfur containing amino acids, it is not surprising that methionine and cystine deficient rats show a retardation in hair growth In this connection it has been reported that vanadium causes a toxicity in rats in which there is early evidence of poor hair growth, comparable to that noted in cystine and methionine deficiency It has been suggested that vanadium may block the conversion of methionine to cystine (Mountain *et al*, 1953)

Alkaptonuria is a disease in which a metabolic block interferes with the conversion of homogentisic acid to acetoacetic acid Homogentisic acid in the urine turns to a black pigment on oxidation In ochronosis a similar black pigment is deposited in the corium of the skin If tyrosine or phenyl alanine is fed to vitamin C deficient infants or guinea pigs, alkaptonuria can be produced and then corrected by giving either vitamin C or folic acid (Levine *et al*, 1941, Morris *et al*, 1950, Sealock and Silberstein, 1940)

Amino acids, especially tyrosine and phenylalanine, are important pre cursors of the skin pigment melanin Melanin is derived from tyrosine by way of 3,4 dihydroxyphenylalanine (dopa) and its oxidation product, 3,4 dioxyphenylalanine (dopaquinone), which is then converted to further melanin precursors Lerner and Fitzpatrick (1950) postulated that a tyro sine deficiency may result in deficient melanin production An individual unable to convert phenylalanine to tyrosine (absence of phenylalanine hy droxylase) would be dependent on dietary tyrosine for melanin formation, such is the case in phenylpyruvic oligophrenia

The pituitary melanophore stimulating hormone (MSH) exerts a power ful effect on melanin formation Lerner *et al* (1953) have shown that injec tions of huge amounts of purified MSH into man produced generalized melanosis and stimulated the appearance of flat, pigmented nevi

Widespread throughout the technically underdeveloped areas of the world, and especially in children between the ages of 1 and 4, is a protein deficiency manifested in the extreme by the disease known as kwashiorkor This disease is characterized by faulty growth, marked liver enlargement with fatty infiltration, gastrointestinal disturbances including diarrhea, and extensive skin changes with hypopigmentation and dryness of the hair and skin (Waterlow, 1955) That the United States is not immune from this disease is indicated by a report of four cases of kwashiorkor from Louisiana (Henington *et al*, 1958)

Williams (1933) in the original description of kwashiorkor described the associated dermatosis and considered it non pellagra in origin The Food and Agriculture Organization (F A O) report of kwashiorkor in Africa (Brock, 1952) concludes that "dermatosis is only an associated condition

which probably may have several different origins and which is not an integral part of the syndrome" The commonest form is described in the following terms "This eruption occurs as sharply defined black varnished patches on the areas exposed to irritation (napkin area, buttocks, back and so forth) but none appears on the areas exposed to sunlight (the hands and face) on which classical pellagra should appear These black islands rapidly enlarge, tend to coalesce and then peel to disclose a white or pink area underneath They undergo spontaneous peeling quite independently of any specific treatment, but this usually occurs only if at the same time the case is improving' A thickened "elephant like skin" may be seen on the exterior surface of the knee

Another interesting feature associated with kwashiorkor is the so called 'flag sign' If the hair is held up, one may see near the base a white band above and below which the hair is of normal color This area of depigmentation represents the period during which the individual had the full blown disease The areas of normal hair color above and below the white band represent the periods before the onset of the disease and after the cure Dyspigmentation may also affect the tips of the hair, with the development of a reddish hue This in part may reflect the influence of exposure to sun and wind

Patchy or diffuse skin dyspigmentation that may be difficult to differentiate from genetic hypopigmentation occurs as a sign of nutritional deficiency in the kwashiorkor syndrome

Therapy for this disease is an adequate amount of protein, particularly of animal origin although mixtures of selected vegetable proteins prove quite satisfactory Mixtures of essential amino acids may also be used in therapy indicating the specific role of protein in the etiology of kwashiorkor (Brock *et al*, 1956) The possibility of supplementing vegetable proteins with essential amino acids to improve their biological value may ultimately be practical as the cost of manufacturing amino acids decreases

III LIPIDS

Several types of skin lesions, eczematous in character, have been associated with the long term consumption of diets low in fat (Hansen and Burr 1946) It has also been noted that individuals who have excessive fat losses associated with idiopathic steatorrhea frequently have erythematosus eczematous lesions

Difficulties arise, however in attempting to correlate these skin lesions with any specific biochemical abnormality For example, while there is no significant difference between the level of total blood lipids in eczematous and noneczematous infants, it has been shown that the amount of unsaturated fatty acids *is considerably lower in eczematous infants*, particularly

the levels of arachidonic and linoleic acids (Hansen, 1937) This observation has encouraged the use of fats and oils rich in these two substances in the treatment of eczema in infants—unfortunately, not with uniformly good results

Studies on the disease phrynoderma, or hyperkeratosis follicularis, have shown that the increased incidence of this disease is correlated with a lowered intake of essential fatty acids Although vitamin A has often been proposed in the treatment of this disease, it has been effective in relatively few cases However, recent studies in India, where this disease is common, have shown that the administration of raw linseed oil or linoleic acid produced marked clinical improvement in 2–4 weeks and cured many patients in 4–24 weeks (Rajagopal *et al*, 1952) Even better results were obtained when pyridoxine was combined with the essential fatty acid therapy of phrynoderma (Bagchi *et al*, 1959)

IV Obesity and Skin Disease

The obese patient seems to be plagued with skin disorders With obesity, one has excessive fat folds with resultant intertrigo, caused primarily by friction between the skin surfaces and by the maceration of the skin from accumulated moisture in these folds These changes often lead to infection, particularly with staphylococcal organisms, or to fungus disease, e g dermatophytosis and moniliasis Obese people, because of their thick subcutaneous layers of fat, tend to become overheated and to sweat profusely This produces undesirable effects on normal skin, exaggerating areas of inflammation and producing skin rashes Prickly heat, or miliaria rubra, is a commonly recognized evidence of sweat retention Obviously, the therapy for this is correction of obesity by decreased caloric intake and increased exercise This is more easily said than done, and the therapy and management of obesity still remain among the most difficult problems facing the physician dietitian, and patient

V Vitamins—Facts and Fancy

The search for vitamins has always captured the imagination of the research scientist, and because deficiencies of these substances are so often associated with skin and hair changes in experimental animals, it was an easy transition from animals to man by dermatologists and physicians eager to treat difficult dermatological problems Hence, vitamins in every form, combination, and dosage level have been used in the treatment of skin diseases As might be expected from this rather haphazard approach, a great variety of results have been reported These are characterized mostly by their inconsistency The question most often raised is the need for vitamins beyond that ordinarily present in a well balanced diet There

is no simple answer to this question, but it should be remembered that in skin disease as with disease in general, there is tissue pathology, presumably accompanying abnormal metabolism Certainly, adequate vitamin intake must be insured, and this is most easily done by appropriate vitamin supplementation The danger lies, however, in overzealous specific claims for "skin cures" effected by vitamins since many dermatological conditions improve without therapy and for no known reason Claims for vitamin therapy must be made only after the closest control of experimental or clinical studies and the most conservative judgment must be exercised

VI Fat soluble Vitamins

1 *Vitamin A*

Since vitamin A is intimately concerned in the maintenance of normal cell structure, one of the most characteristic changes seen in vitamin A deficiency is disintegration of epithelial surfaces followed by their replacement with a keratinized stratified epithelium

In man lack of vitamin A (Wohl and Goodhart, 1960) may result in dryness scaliness, furunculosis and abscesses of the scalp and in bleaching drying out or loss of the hair The follicular lesions, which are most helpful in establishing diagnosis vary in size, the maximum diameter being 5 mm They are hard deeply pigmented, and surrounded by a zone of pigmentation The center of the lesion, the papule, is a scaly, pointed plug of keratinized epithelium which, if pressed out, leaves a crater Though comedones are common on the face, the keratinized lesions do not occur there, hence the two are never associated, though both respond promptly to treatment with vitamin A Histologic examination shows hyperplasia and hyperkeratinization of the related epithelium around the hair follicles, together with metaplastic changes of the sweat ducts and degeneration of the glands, accounting for the dryness of the skin

Frazier and Hu (1931) have described a skin eruption occurring in Chinese soldiers which they consider typical of vitamin A deficiency The skin first becomes dry and rough, then spinous papules appear at the sites of the hair follicles—first on the anterolateral surfaces of the thighs and the posterolateral aspects of the arms, at last the eruptions occur over the entire integument In debilitated patients and in those with poor habits of personal cleanliness pustules may develop Occasionally, atrophic ulcers appear Microscopic study of the tissues shows hyperkeratinization of the epidermis and follicles together with metaplasia of the sweat duct epithelium into that of the squamous type Proper diet causes a gradual improvement

As might be anticipated, those skin lesions which are associated with a deficient intake of vitamin A respond to therapy with this vitamin

and Butler, 1938) which is manifested by a sore, magenta colored tongue, cheilosis, angular stomatitis, and seborrheic dermatitis about the nose and scrotum Horwitt (1955a) reported on experimental riboflavin deficiency in human subjects and emphasized that all features of the riboflavin syndrome do not necessarily appear in all subjects Local trauma, irritation, or infection may serve as a trigger mechanism to make manifest the signs of deficiency

Of interest are the results of studies on nutrition in the Far East by Pollack (1956) This work, carried out on Chinese Nationalist Army troops on Formosa, indicates a high incidence of riboflavin deficiency, and an interesting associated syndrome called the oral genital syndrome has been described Scrotal dermatitis was observed very often in those individuals who also had angular stomatitis, cheilosis, magenta tongue, and nasolabial seborrhea When the biochemical findings in blood and urine were correlated with the clinical findings in the oral genital syndrome, it was evident that a riboflavin deficiency was involved Niacin deficiency was also noted in significant numbers in this study Enrichment of the rice in the diet with riboflavin and niacin rapidly eliminated the signs attributable to the deficiency of these vitamins It is now becoming evident from nutrition surveys conducted in many areas of the world that riboflavin deficiency is one of the most common

2 *Niacin Deficiency—Pellagra*

The earliest symptoms of pellagra may involve either the skin or the gastrointestinal tract Premonitory evidence of skin involvement may appear as a temporary redness like that of sunburn, it clears up without a trace only to return later in severer degree Earliest lesions are usually macular, of a light or dark red color, but these coalesce and form a dark red or purplish eruption followed by desquamation (Spies, 1955b) The areas involved are those of friction and exposure The face, neck, hands, forearms, and feet are the usual sites This early eruption may be accompanied by considerable swelling of the involved parts In severe cases, bullae may be present, but usually these dry up, leaving crusts, though they may of course become infected Ulceration has been known to occur A highly characteristic feature of the erythema and subsequent pigmentation is the occurrence of sharp margination of the wrist or forearm producing the so called "pellagrous glove " The skin manifestations are usually present in three stages (1) congestion, (2) thickening and pigmentation, (3) atrophic thinning Subjective symptoms are usually slight, and they consist chiefly of a burning sensation rather than an itching

The mucous membranes are also involved The tongue becomes swollen and denuded and is often brilliant red in color The buccal mucous mem

brane may, in severe cases, have a similar appearance, the redness may at times extend to the lips too Sometimes the oral commissures become fissured

Thousands of pellagrous patients have now been treated with niacinamide Doses up to 3 or 4 gm daily may be administered safely, although the average dose is 100 mg three times daily In a day or two, the redness of the oral pharyngeal, and vaginal mucous membranes is reduced, nausea, vomiting, and excessive salivation decrease, and bowel movements become normal Unless the continuity of the skin is broken, the acute red lesions fade rapidly The acute mental symptoms, varying from confusion to delirium and mania, usually disappear quickly The addition of thiamine is often necessary to improve symptoms due to involvement of the peripheral nervous system Riboflavin may be required if there is clinical evidence of an associated riboflavin deficiency This is indicative of the multiple nature of deficiency diseases

Diets in which corn predominates are prone to lead to niacin deficiency and pellagra, especially if these diets are inadequate in proteins that contain tryptophan (Krehl *et al*, 1946) The niacin requirement is significantly affected by the amount of tryptophan in the diet, as this amino acid is metabolically converted by the body to niacin The approximate dietary replacement ratio is 50 or 60 mg of tryptophan to 1 mg of niacin (Horwitt, 1955b)

Another interesting aspect of niacin therapy is the dermatological effect of producing a marked cutaneous vasodilatation with extensive flushing of the skin This characteristic is not shared by niacinamide

Many of the phenomena observed in pellagra, such as abdominal distress diarrhea, pigmentation of the skin and photosensitivity are often found in patients exhibiting acute toxic porphyria Rosenblum and Jolliffe (1940) found porphyrinuria present in six of nine inebriates who had pellagra In only three of these could the porphyrinuria be correlated with the severity of the disease It was concluded that the symptoms of pellagra were in no way dependent on impaired porphyrin metabolism as manifested by increased porphyrinuria The presence of liver dysfunction in the patients studied may well have accounted for the excessive production of coproporphyrin III

3 *Vitamin B_6 (Pyridoxine, Pyridoxal Pyridoxamine)*

Animals especially the rat develop severe skin lesions in vitamin B_6 deficiency (Gyorgy and Eckhardt 1939) Vasodilatation with erythema over the skin of the paws, followed by hyperkeratosis scaling and edema occurs Essential fatty acid deficiency results in a dermatitis in the rat similar to that seen in pyridoxine deficiency This is indicative of an inter

relationship between pyridoxine and fatty acid metabolism High fat diets, rich in the essential fatty acids, will delay the onset of pyridoxine deficiency dermatitis, and the reverse of this situation also holds true (Gross, 1940) Vitamin B_6 is important particularly for the conversion of linoleic acid to arachidonic acid

Vitamin B_6 is essential also in the metabolism of tryptophan, and this fact is used as a test for the adequacy of pyridoxine nutrition After a test load of tryptophan, the urinary excretion of xanthurenic acid, an abnormal metabolite of tryptophan, is greatly increased if the individual has had a previous low intake of vitamin B_6 (Lepkovsky *et al*, 1943) Pyridoxine deficiency has been produced in human subjects (Schreiner *et al*, 1952) by the use of a pyridoxine antagonist 4 deoxypyridoxine, and in association with this deficiency there is seen a seborrhea like lesion about the eyes, nasal labial fold, and mouth, with extension to the eyebrow and skin behind the ears The scrotal and perineal regions were occasionally involved Intertrigo developed under the breasts and in other moist areas Hyper pigmented and scaly pellagra like dermatitis also developed in some in stances in the regions of the collar, forearms, elbows, and thighs There was a significant increase in xanthurenic acid excretion after a test load of tryptophan There was noted a rapid regression of all lesions, including a reduction in xanthurenic acid excretion, after the administration of pyri doxine, pyridoxal, or pyridoxamine in doses as low as 5 mg daily (Vilter, 1956) Variable success has been claimed in the treatment of ordinary seborrheic dermatitis using a pyridoxine ointment in topical application, suggesting the possibility of a local vitamin B_6 metabolic defect in the skin

4 Pantothenic Acid, Inositol, p Aminobenzoic Acid, and Biotin

Experiments reported by Woolley (1940a) have shown that pantothenic acid has an influence on the growth of hair It has also been shown that a deficiency of pantothenic acid is a factor in the graying of hair and that pantothenic acid improves the skin and decreases graying of the fur of experimental animals (Dimik and Lepp, 1940, Morgan and Simms 1940) Unna *et al* (1941) found that black rats fed a synthetic diet deficient in pantothenic acid become gray in from 4 to 6 weeks Pantothenic acid at a level of 100 mg a day will prevent or cure this condition On the other hand, Williams and Major (1940) found pantothenic acid to be without effect on the graying of hair There is no evidence that pantothenic acid acts in hu man beings as it seems to act in various animals Spies *et al* (1940) believe, however that pantothenic acid and riboflavin are intimately associated in their behavior, and it is true that patients with cheilosis and other mani festations of riboflavin deficiency have shown marked improvement when given pantothenic acid Though the human requirements and possible

therapeutic doses are unknown, it is known that as much as 100 mg of pantothenic acid daily is well tolerated

Little is known about inositol, its relation to human nutrition is still a subject for investigation Considerable interest has been aroused by the investigations of Woolley (1940b), who has shown that inositol will cure alopecia in mice (Woolley, 1941)

Thirty three patients with lupus erythematosus were given 1–4 gm of p aminobenzoic acid (PABA) at 2–3 hour intervals by Zarofonetis (1949) Two of ten with chronic discoid lupus showed no improvement, one patient had a poor response, and seven had good to excellent responses Improvement occurred in the seven patients with scleroderma the sclerodermatous areas gradually softening and becoming thinner and more pliable PABA produced improvement in five patients with dermatitis herpetiformis Toxic hepatitis, drug fever and nausea and vomiting may occasionally result from PABA, and much more careful work must be done before any serious recommendations can be made for its use in dermatological conditions

Biotin deficient rats develop marked loss of hair with erythema of the underlying skin Marked periorbital hair loss is also noted, which may represent in part a complication from inositol deficiency No counterpart deficiency has been demonstrated in man

IX Side Effects of Vitamin Therapy

Certain undesirable cutaneous side effects may be associated with the use of vitamins These are for the most part allergic in character Thiamine may cause urticaria angioneurotic edema, pruritis, and contact dermatitis Niacin produces marked erythema, peripheral vasodilation, and pruritis This is a common reaction in patients who are given niacin in an attempt to lower the blood cholesterol This cutaneous reaction to niacin, interestingly is not seen with niacinamide, which incidentally, does not lower blood cholesterol levels

Vitamin B_{12} which contains cobalt, may cause reactions in cobalt sensitive patients especially when given by the parenteral route

Vitamins, like other chemical substances, might be expected to cause allergic reactions with dermatological manifestations in a certain number of individuals Fortunately, the incidence is insignificant

X Minerals and Trace Elements

A large number of mineral elements have been shown to be essential nutrients, either in macro or micro amounts Animals fed a diet deficient in any one of four of these elements, i e, calcium magnesium zinc, and copper, develop skin lesions

1 *Magnesium*

Magnesium deficiency in the rat (McCollum and Orent, 1931) produces very dramatic changes not only in greatly increased nerve and muscle irritability, but also in the skin (Sullivan and Evans, 1944a) After about 7–12 days on a magnesium deficient diet, marked erythema of the skin develops This is a transitory phenomenon, and in a matter of a few minutes the animal's skin may change from a flushed, erythematous condition to a pale, white, bloodless appearance As the deficiency proceeds, the erythema is more persistent, at the same time irritability increases to the point where convulsive seizures may develop on the slightest stimulation The skin changes continue with the development of edema, scaling, and hyperkeratosis Although serum magnesium levels are reduced, the magnesium content of the skin remains normal (Sullivan and Evans, 1944b) Curiously, in magnesium deficient rats, nucleated red blood cells are seen in abnormally large numbers in the peripheral blood (Krehl, 1957) The fact that magnesium is an essential factor in the functioning of a number of important enzymes is indicative of its importance in normal metabolism

2 *Calcium*

It has been suggested that calcium has an important function in capillary permeability through its effect on the calcium proteinate of the intercellular cement substance (Chambers and Zweifach, 1940) Calcium deficient animals develop extensive hemorrhages supposedly because of the weakness of capillary walls lacking in extracellular cement substance

3 *Zinc*

Todd *et al* (1934) produced zinc deficiency in the rat, observing growth retardation, alopecia, dryness and scaling of the skin, all of which could be corrected by the addition of 0 1 mg of zinc to the daily diet Follis *et al* (1941) demonstrated histologically the presence in the skin of acanthosis and heavy parakeratotic scales Also noted was a marked atrophy of the hair follicles while the sebaceous glands showed a distinct hyperplasia

4 *Copper*

Copper deficient animals exhibit a severe anemia, not correctable with iron, with poor growth and skin and hair changes (Hart *et al*, 1928) Copper has an unusual effect on the formation of disulfide cross linkages (Marston, 1952) The wool fibers from copper deficient sheep are weak because of artificial rupture of disulfide linkages Also, the total sulfur content of wool fibers is 10–15% below normal in copper deficiency It is thought that the oxidative closure of the sulfhydryl residues to disulfide linkages is catalyzed by copper Copper is also essential for tyrosinase activity and hence is of importance in melanin formation (Lerner *et al*, 1949)

XI Diet and Dermatological Problems

1 Allergy

Food allergy may be the cause of many skin lesions, including atopic dermatitis, urticaria, infantile dermatitis, and eczema (McGovern and Zuckerman, 1956

The allergenic properties of a food depend on the nature of the food, the quantity and concentration of the food in the diet, and the duration of exposure to the allergenic substance In addition, one must consider the problem of host susceptibility A family with an allergic background should alert one to exert special care in the feeding of its infants and small children, particularly during the period of 'physiologic, immunologic immaturity "

Food allergens may enter the body by way of the gastrointestinal tract, and derangements of the gastrointestinal system are among the prime factors leading to food allergy

Almost all foods and beverages have at one time or another been incriminated as being allergenic, but those that are most common are unaltered egg protein, milk protein, fish and seafood wheat, corn, tomatoes, spinach, cabbage, asparagus rhubarb, celery, onion, garlic, and citrus fruits and juices

When the allergenic role of a food has been established it may be corrected by elimination of the food in question or by specific desensitization Unfortunately food elimination as a means of therapy may lead to rather bizarre and unusual dietary patterns There must be careful supervision of the diet under these circumstances in order to insure adequate and balanced nutrition

To indicate the really great difficulties and complexities of dermatology, two important dermatological problems with unknown etiology and for which there is no specific proved therapy deserve mention These are psoriasis and acne vulgaris These are selected also because diet has been so extensively involved in their management

2 Xanthoma

Xanthoma of the skin, characterized by round or oval, solid firm reddish brown papules, are often distributed over the buttocks, elbows, and knees and may be seen in patients with diabetes or hypercholesterolemia from almost any cause, a familial form of xanthomatoses is also known Analyses of these xanthomatous lesions show an excess of fatty acids, cholesterol, and other lipid substances Their deposition probably reflects the high cholesterol content of blood and other lipids in patients with disordered fat metabolism such as may be seen in poorly controlled diabetics and in patients with other abnormalities of lipid metabolism, some of which may be familial in origin Some observers have reported marked improvement

and even disappearance of these xanthoma when the patient was placed on a low fat diet for a long period of time (Wile *et al*, 1929) Unfortunately, this is often very difficult to achieve and the results are discouraging

3 Psoriasis

Psoriasis is a chronic, inflammatory skin disease characterized by the development of reddish, erythematous patches covered with raised, silvery white, dried scales Cracking and fissuring of the skin in the extensive psoriatic area is quite common The disease affects primarily the extensor surfaces of the body and the scalp, although it may be very widely disseminated

Although such patients have failed to show any clear cut metabolic distubance as the underlying cause of this dermatitis, some interesting studies have been conducted It has been demonstrated that a diet free from fat or very low in protein is moderately effective in the treatment of psoriasis (Buckley 1912) Schamberg (1932) has been the outstanding proponent of the low protein diet and he has indicated that psoriatic individuals give definite signs of abnormal nitrogen retention Patients with psoriasis, placed on diets containing no more than 4 or 5 gm of nitrogen per day, with an adequate caloric intake, were able to maintain their weight and experienced a gradual disappearance of the lesions of this disease Unfortunately, when this very restricted dietary regimen is discontinued, the disease returns

Psoriatic scales containing keratin like protein have an unusually high content of sulfur amino acids In this connection, it is worth noting that the standard therapy of psoriasis with coal tar and ultraviolet light are both known to disrupt the metabolism of the sulfhydryl group and may provide a clue to understanding and therapy of this disease It has also been reported that there is an interesting relationship between decreased urinary *secretion of sulfur and of ascorbic acid in patients with psoriasis* Experiments with diets containing a maximum of 250 mg of sulfur per day in patients for periods of several months have yielded inconsistent results It would be of considerable interest to feed psoriatic patients for extended periods of time on purely synthetic diets with known intakes of all nutrients In this way, the role of the sulfur amino acids metabolism in psoriasis might be clarified

4 Acne vulgaris

Acne vulgaris is a chronic inflammatory disease of the sebaceous glands and the pylosebaceous structures of the skin and is almost always associated with seborrhea This is predominantly a dermatological disease of youth, particularly teenage youth, and associated with it are all the emotional and psychological factors so prevalent in this age group It is a

rather common clinical experience that gross dietary indiscretion, particularly by taking too much cake, candy, ice cream and chocolates aggravates an existing acne This strongly suggests that the skin condition may be associated with a disturbance of carbohydrate metabolism (Lemon and Hermann, 1940) Not all agree on this, and improvement has been reported in patients taking a high carbohydrate diet (Crawford and Swartz, 1936)

It is also thought that acne is made worse by diets high in fat Unfortunately, low fat diets have not been particularly effective in the management of this problem It may well be that the principal effect of high fat diets is to permit excessive weight gain in individuals with acne, who are often withdrawn, quiet, and relatively inactive because of the emotional trauma that is associated with this disease It has been pointed out that the diet of the Eskimo is mostly fat and protein, but Eskimos have relatively little or no acne One cannot doubt that psychoneurogenous factors play a significant role in producing exacerbation of this disease In addition, fatigue and exhaustion are often predisposing factors

In view of the fact that we have no specific knowledge as to the cause or treatment of acne it seems most reasonable to place people with this disease on a well balanced diet that does not contribute an excessive amount of calories, so that weight gain is not excessive Such a diet should contribute all the food nutrients in the recommended daily allowances Perhaps most important of all, the child or individual with this disease, as with all difficult dermatological diseases, must have the necessary psychological lift from the physician and from his friends and family to permit him to learn to live with his disease and function as much like a normal individual as possible Again often easier said than done

XII Summary

There is little doubt that dietary deficiency, whether primary or conditioned may be associated with dermatological lesions that can respond to specific therapy with vitamins or unusual diets

Dermatoses are certainly associated with obesity, and since this is such a common disease therapy directed at weight reduction will probably provide the best management in related dermatological problems

The general dermatological changes associated with undernutrition, rather rare in this country, are extremely common in technically underdeveloped areas, and here improvement of the protein intake seems to be the most rational way of correcting these difficulties Along with increased protein intake would come more vitamins

Certainly, allergies to foods may produce dermatoses and such allergies should be ruled out if suspected either on the basis of family history or by history of the patient s food intake

It is extremely important that careful nutritional history should be taken

on every patient presenting a dermatological problem and that phys examination and, if necessary biochemical studies should be made in or to rule out nutritional abnormality

For example, phenylpyruvic oligophrenia may be diagnosed simply early by testing the urine with ferric chloride Early diagnosis and thera by restricting phenylalanine will produce most gratifying results

It must be remembered that the underlying defects related to dermato of unknown etiology may be the result of improper nutrition or metab errors endured over very long periods of time Hence, their correction diet or improved metabolism may require long and intensive therapy

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The Neuroendocrine System of Arthropods*

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These authors, in agreement with other earlier studies, found a single or paired median group of neurosecretory cells in the pars intercerebralis and one or two lateral groups of cells as well The question of the number of types of cells to be found in this region of the brain has been much discussed, and there is currently no agreement among individuals working with various species, figures range from one to six types of cells On the available evidence, it seems likely that there are several types and that only careful studies using criteria of anatomical localization, secretory cycles, and staining or histochemical properties will permit development of generalities concerning the number of distinctive neurosecretory cells in any region The correlation of histological with physiological studies should prove especially rewarding

The neurosecretory tracts from the cells in the brain have been quite clearly traced in many species of insects (Arvy, 1956, Arvy and Gabe, 1954a, b, DeLerma, 1956, Dupont Raabe, 1956a,b,c, 1957, Gabe, 1954a, M Thomsen, 1954a b) The axons from the cells in the pars intercerebralis typically run anteriorly, some or all cross to the opposite side of the brain to form paired tracts which run posteriorly to emerge from the posterior face of the protocerebrum as the nervi corporis cardiaci I The axons of the lateral neurosecretory cells run posteriorly without crossing and emerge near the roots of the first pair of nerves as the nervi corporis cardiaci II Both pairs of nerves are typically short and innervate the corpora cardiaca, paired endocrine structures to be discussed later Dupont Raabe (1956a,b,c, 1957) has described a third cardiac body nerve in *Carausius morosus*, probably arising from the tritocerebral neurosecretory cells This appears to be exceptional, but has also been described in *Oncopeltus fasciatus* by Johansson (1958b)

Many of the axons of the nervi corporis cardiaci terminate within the corpora cardiaca, but some pass through without interruption and emerge as the nervi corporis allati, innervating the corpora allata, paired or fused endocrine glands posterior to the corpora cardiaca Histological evidence from 1940 onward (Gabe 1954a) suggested that the neurosecretory product moves along the axons from the cell bodies to the endings in the corpora cardiaca and allata Direct evidence of such movement in living cells of *Calliphora erythrocephala* (Diptera) was obtained by E Thomsen (1954a,b) Possompès (1958) has provided experimental evidence in the same species that section of the nerves prevents the physiological action of the brain cells, presumably by interfering with axonal transport

Little attention has been given to the neurosecretory cells in other parts of the central nervous system B Scharrer (1955a,b 1956) has distinguished three cell types in the subesophageal ganglion of *Leucophaea maderae* (Orthoptera) and has shown a functional relation to reproduction in one

type Nayar (1956b) reports that the axons of the recurrent nerve of the stomatogastric nervous system of *Iphita limbata* (Hemiptera) carry neuro secretory material, probably from the hypocerebral ganglion and the brain, and that this material may be discharged directly into the aorta from nerve endings located there

b Arachnids Diplopods and Chilopods The complete lack of histological information concerning neurosecretion in arachnids which Gabe (1954a) noted earlier no longer exists, thanks to the studies of Gabe (1954b,c), Legendre (1953, 1954a,b, 1956, 1958a b), Kuhne (1957), Herlant Meewis and Naisse (1957), and Naisse (1959) In general there are two pairs of neurosecretory cell groups in the protocerebrum, anterior and posterior respectively In the phalangids Naisse (1959) has also noted a lateral pair, which appears only in the third instar and begins to secrete later There are also neurosecretory elements in the subesophageal pedal, abdominal, and pedipalp ganglia The axons of the protocerebral cells run in the sto matogastric nerves described by Schneider in 1887, these nerves supply two pairs of stomatogastric ganglia which Legendre calls the organs of Schneider Kuhne (1957) disagrees with Legendre's description of the innervation of these organs There is histological evidence of axonal trans port of the neurosecretory material from the protocerebral cells

Diplopods (millipedes) and chilopods (centipedes) have protocerebral neurosecretory cells which send axons to lateral end organs or ganglia, and axonal transport has been observed here There are also neurosecretory cells in the tritocerebrum, subesophageal ganglion and ventral nerve cord (Gabe 1954a,c, 1956a, Palm, 1956, Sahli, 1958)

c Crustaceans The study of the crustacean neurosecretory system has, for many years, been concentrated on the X-organ sinus gland system of the eyestalk of decapods This concentration arose out of the experimental evidence of Koller (1928) and Perkins (1928) implicating the eyestalk in the humoral control of color change, followed by the description of Han strom (1931) of the X-organ and the sinus gland, and his later proof (1935) that the region of the sinus gland contains the highest concentration of chromatophorotropic activity During the last five years, morphological studies have made it clear that the neurosecretory system is much more complex than was previously supposed, and the results of these studies make it desirable to revise our nomenclature, our thinking and our experi mental procedures in the study of crustacean endocrinology

The general pattern of organization of the crustacean central nervous system differs from that of the insects in that the brain extends in most forms, into two optic peduncles, more or less separated from the rest of the brain and including three primary optic centers, the medulla interna, medulla externa and medulla terminalis Recent studies have distinguished

three to six groups of neurosecretory cells in the eyestalks, and five or six in the supraesophageal ganglion or brain proper, neurosecretory cells are also found in the ventral nerve cord Several of the cells in both eyestalk and brain send axons to the sinus gland, an end organ in the eyestalk (Bliss *et al*, 1954a,b, Fingerman and Aota, 1958a, 1959, Carlisle, 1959b) The sinus gland thus receives three to five distinct kinds of axon termini, which can be distinguished on histological and histochemical criteria (Potter, 1954, Rehm, 1959) Axonal transport of neurosecretory material has been demonstrated in fixed and living preparations of some of the cells, particularly those with cell bodies in the medulla terminalis and axon termini in the sinus gland (Enami, 1954, Passano, 1954a, Matsumoto, 1958, Fingerman and Aota, 1958b)

The question of the nomenclature of the neurosecretory cell groups in the eyestalk has been discussed extensively by Knowles and Carlisle (1956) Carlisle and Knowles (1959), and Carlisle (1959b) It seems to the present author that most of the difficulties which have arisen could be eliminated by abandoning altogether the term "X organ" The structure which Hanstrom (1931) designated thus could be referred to as the organ of Bellonci in isopods and stomatopods, the organ of Hanstrom in decapods, or in general as the sensory papilla organ, since it occurs usually in association with a sensory papilla or pore The neurosecretory cell groups in the eyestalk could be designated by reference to the ganglionic mass in which they occur, as medulla interna, medulla externa, or medulla terminalis cell groups This nomenclature would be in accord with anatomical practice, would simplify the somewhat more cumbersome terminology introduced by Carlisle, and avoid the confusion between neurosecretory cell groups and their corresponding end organs which has arisen from the practice of referring to the medulla terminalis cell group, in certain crustaceans, as the X organ

The cytology of the neurosecretory cells has been studied by Enami (1954), Matsumoto (1954a,b, 1956, 1958, 1959), Durand (1956), and Parameswaran (1955, 1956) From their work, it is quite clear that there are several distinct cell types, on the basis of anatomical localization staining properties, and secretory cycles Matsumoto whose study was the most complete, distinguished as many as eleven types Some of these are confined to a single neurosecretory cell group, others are found in several regions of the central nervous system In any one neurosecretory cell group, there are generally several distinct cell types

d Conclusions We have seen that all the major groups of arthropods have neurosecretory cells distributed through the central nervous system in more or less discrete groups Among these cells are a number of types, distinguishable on the basis of staining properties, localization and pattern

of secretory activity A fruitful area of comparative study appears in the careful evaluation of the cell types in the different species and, if possible establishment of homologies among them As we shall see later correlation of the secretory cycles with various cycles of vital activities may provide valuable physiological information Some of the cells send axons to definite end organs, and clear evidence has been obtained from histological and experimental studies and from direct observation of the living cell, that the secretory product is transported along the axons to the end organ and liberated from the axon terminals either among the cells of the end organ or directly into the circulating fluid The most striking such neurosecretory system among the arthropods consists of several groups of neurosecretory cells in the protocerebrum, which send axons to the end organs known variously as lateral glands, Schneider organs, the cardiacum allatum complex, or the X organ sinus gland complex The parallel with the hypothalamus neurohypophysis system of the vertebrates is evident In all the arthropods, however, there are important aggregations of neurosecretory cells in the tritocerebrum, the ventral ganglion chain, and the accessory ganglia which do not send axons to these end organs

2 The Endocrine System

Most of the structures which we shall consider in this section as endocrine glands are in part at least, end organs for the neurosecretory system In recent years two distinct endocrine structures with no direct connections to the neurosecretory system have been discovered, these are the thoracic gland, ventral gland or molt gland of the insects and crustaceans and the vas deferens gland or androgenic gland of certain crustaceans

a Insects The known endocrine organs of insects are the corpora cardiaca, the corpora allata and the thoracic gland The first two of these are neurohemal organs lying in or on the wall of the aorta In the dipterans, all three structures are closely associated in or near the ring of Weissmann or the ring gland (cf Fraser, 1955)

1 Corpora cardiaca These are typically paired organs on or in the lateral walls of the aorta except in the Diptera, where the cardiacum cells are incorporated in the ring gland (Clements, 1956) Embryologically they are modified sympathetic ganglia arising with the hypocerebral and the ventricular ganglia from an unpaired dorsal invagination of the esophagus (Highnam, 1958a, Novak, 1959) They are found in all major orders of insects with the possible exception of Collembola (Novak, 1959)

The corpora cardiaca receive axons from the protocerebral neurosecretory cells as noted above In addition to the axon termini there are intrinsic cells usually of two types Some cells are large, usually located peripherally, phloxinophile, and sometimes syncytial or with pseudopodial extensions

(DeLerma, 1956, Dupont Raabe, 1956b,c, 1957, Highnam, 1958a, Grandori and Care, 1954, Lhoste, 1957, Meyer and Pflugfelder, 1958, Nayar, 1954, M Thomsen, 1954a,b) The secretory granules of these large cells stain differently from the granules in the axon termini, and the secretory cycle of the intrinsic cells is different from that of the termini It seems probable that the large cells form an intrinsic product distinct from that of the neurosecretory cells (Arvy, 1956, Gabe, 1954a, Grandori and Care, 1954, Herlant-Meewis and Paquet, 1956) Other cells are small, chromophile, and not invariably present, they appear relatively late in development in some insects at least (Formigoni, 1956, Johansson, 1958b, Lhoste, 1957, Nayar, 1954, M Thomsen, 1954a) The neurosecretory axon termini appear to penetrate deeply into these cells, reaching as far as the nuclear membrane in some cases (Formigoni, 1956, Meyer and Pflugfelder, 1958), it is possible that the small cells correspond to glial cells (Johansson, 1958b)

ii Corpora allata The corpora allata may be single or paired, and are generally more or less closely associated with the corpora cardiaca, to which they are connected by the nervi corporis allati As we noted earlier, the axons in these connectives originate in the protocerebral neurosecretory cells and pass without interruption to the corpora allata The passage of neurosecretory product from the protocerebrum to the corpora allata has been demonstrated in several species of insects (Gabe, 1954a, Nayar, 1956a) In the Plecoptera studied by Arvy and Gabe (1954a,b), there is a nerve from the corpus allatum to the thoracic gland and neurosecretory material was seen in the axons of this nerve This appears to be exceptional Highnam (1958b) also noted axons extending from the corpora allata of *Mimas tiliae* toward the aorta Johansson (1958b) and Engelmann (1957) note that the corpora allata are innervated from the subesophageal ganglia as well as from the brain

Embryologically the glands originate as invaginations of the ectoderm at the boundary between the mandibular and maxillary segments They are present in all higher insects and in rudimentary form in some Apterygota (Novak, 1959) The histology varies considerably, and Novak (1959) distinguishes four types of glands in different groups of insects on the basis of shape and cell structure In recent studies Nayar (1956a) describes the corpus allatum of *Iphita limbata* (Hemiptera) as syncytial with two kinds of nuclei The secretory cells of the corpus allatum of *Mimas tiliae* are large and do not have distinct cell boundaries The gland also contains cell that are identified as neurons These are peripherally located with axons extending to the interior of the gland and have not been noted previously (Highnam, 1958b)

There is no doubt that the corpora allata have an intrinsic secretory activity, although this is not always evident from simple histological

criteria (Gabe 1954a, M Thomsen, 1954b, Formigoni, 1956, Lhoste, 1957 Novak, 1959) The secretory cycle is connected with developmental processes and with reproduction, and involves marked changes in volume of the gland which result from changes in cell size and number (von Harnack and Scharrer 1956, B Scharrer and von Harnack 1958 von Harnack, 1958a,b, Formigoni, 1956, Highnam, 1958b)

iii Thoracic gland This structure, known in various insect groups as the ventral gland, prothoracic gland, pericardial gland and peritracheal gland, is the only known endocrine organ in insects which is structurally dissociated from the neurosecretory system Wells (1954) has reviewed briefly the history of its discovery and suggests that the most suitable general name is thoracic gland He describes the structure in eleven species of Hemiptera heteroptera Typically in these and in other insects it consists of a rather loose and extended aggregation of cells in the thoracic region Embryologically it is closely associated with the salivary gland The thoracic glands develop from one of two pairs of lateral invaginations on either side of the second maxillary segment One of these becomes the salivary glands and the other becomes the thoracic glands In most hemipterans the thoracic glands elongate as the salivary glands grow, and the former become a long rope of cells extending between the prothoracic spiracle and the anterior end of the salivary gland In some hemipterans, however, the gland is finally represented merely by a group of nuclei in the fat body In *Apis mellifica* the gland is much broken up and diffuse (Schaller, 1955) In coleopteran larvae the thoracic glands are associated with major tracheal trunks (Nuñez, 1954 Srivastava, 1958, 1959) The microscopic structure may be syncytial or cellular and there is no systematic regularity to this or to the number of cells, which may range from about 10 to more than 500 The gland appears to be present in all groups of insects In the Pterygota it generally atrophies after the imaginal molt (Novak, 1959) It is not normally innervated, the Plecoptera studied by Arvy and Gabe (1954b) are exceptional in this respect

b Arachnids The general endocrine arrangements in arachnids seem to be rather similar to those of insects The Schneider organs of araneids have an anatomical relation to the brain and nervous connections which resemble those of the corpora cardiaca and allata, but extensive considerations of homology seem premature in the absence of embryological evidence There is also a gland in the head which, in structure and secretory cycle, resembles the thoracic gland of insects (Gabe, 1954a, Legendre 1953, 1954a 1958b, Herlant Meewis and Naisse, 1957, Naisse 1959, Kuhne, 1957)

c Crustaceans The known endocrine organs of crustaceans are the sinus gland, the sensory papilla organ the postcommissural organ, the ventral gland (Y organ) and the vas deferens gland (androgenic gland)

Of these the first three are neurosecretory end organs and there is little evidence for an intrinsic endocrine function. The ventral gland is clearly homologous with the thoracic gland of insects, but the vas deferens gland is found only in crustaceans.

i. Sinus gland. When this structure was first described by Hanström (1933), and for almost twenty years thereafter, it held the distinction of being the only known endocrine gland in the crustaceans. In 1952, however, it became evident that the gland is a neurosecretory end organ and it was seriously questioned whether it had an independent endocrine role (Knowles and Carlisle, 1956; Carlisle and Knowles, 1959). Passano (1954b) and Bliss *et al.* (1954a,b) in their important studies of the relation of the sinus gland to the neurosecretory system, considered the gland simply as a site for storage and release of the neurosecretory product. Gabe (1954a), on the other hand, regards the sinus gland as comparable with the corpora cardiaca of insects. There is clearly no question of homology, since the sinus gland originates as a thickening of the epineurium of the optic ganglia and is then invaded by the neurosecretory axons (Pyle, 1943; Matsumoto, 1958), whereas the corpora cardiaca, as we have seen, arise with the other accessory ganglia from the wall of the embryonic esophagus. The observation of Enami (1954) and Bliss *et al.* (1954a,b) that a cut sinus gland nerve will regenerate at its central end a structure resembling a sinus gland emphasizes the *significance of the gland as an end organ.*

Gabe (1954a) rests his case for an intrinsic secretion in the sinus gland on the presence in the gland of a secretion distinct in staining properties from that of the neurosecretory cells and on the presence of nucleated cells in the gland. The fact that the gland receives four to six kinds of axon termini (Potter, 1954; Carlisle, 1959b) means that there will be present several distinct neurosecretory products among which it might be difficult to detect with certainty an intrinsic product. Hodge and Chapman (1958), using the electron microscope, find nonnervous cells in the sinus gland of *Gecarcinus lateralis*. Carlisle could not find such cells in *Lysmata seticaudata* (Knowles and Carlisle, 1956), but he reports the presence in the sinus gland of *Pandalus borealis* of no more than twenty five nuclei which he attributes to connective tissue cells (Carlisle, 1959b). At the moment the evidence favors the view that the sinus gland is a neurohemal organ without intrinsic secretory function, but further study is needed.

ii. Sensory papilla organ. This structure when it occurs outside the optic lobe ganglia includes endings of neurosecretory cells from the brain and optic lobe ganglia, secretory cells of an epithelial type, neurosecretory cells in some species, sensory cells of the sensory pore or papilla and the characteristic concentrically layered "onion bodies" (Knowles and Carlisle, 1956; Carlisle and Knowles, 1959). The onion bodies are interpreted as

accumulated neurosecretory product, and Carlisle (1959b) has described cyclical variations in these deposits in *Pandalus borealis* He gives no evidence of any intrinsic secretory activity in the cells of the organ itself, and there is no indication that it has any role other than that of a neuro secretory end organ

iii Postcommissural organs Knowles (1954), Knowles and Carlisle (1956), and Carlisle and Knowles (1959) have given considerable attention to a pair of small neurohemal organs on the tritocerebral commissure of natantian crustaceans They contain neurosecretory material, but the cells of origin have not been identified There is no evidence of any intrinsic secretion and the organs have not been found in crabs

iv Ventral gland This structure was described briefly by Gabe in 1953 and somewhat more fully later (Gabe, 1956b) In his initial description, Gabe used the provisional name "*organe Y*" and emphasized morphological and embryological similarities to the thoracic gland of insects He and his associates have since provided evidence of a functional analogy which will be discussed later but which leaves no doubt that the ventral gland of crustaceans is both homolog and analog of the thoracic gland of insects The ventral gland was seen as early as 1888 and regarded as an atrophic kidney Gabe has found it in all the 53 species of malacostracans which he had examined in 1953 In those groups, including decapods, with an antennary kidney the gland is in the maxillary metamere In the Isopoda, Stomatopoda and a few others the gland is antennary and the kidney maxillary The gland is located close to the body wall in association with the ventral hypodermis and the designation "ventral gland" seems most appropriate Gabe's suggestion that it be called "molt gland" deviates from the widely accepted practice of naming structures on an anatomical rather than a physiological basis The provisional designation "Y organ" should be abandoned as soon as possible if for no other reason than that an alphabetical system of nomenclature which starts so near the end of the alphabet has obvious limitations

The form and cell structure of the ventral gland vary greatly The gland is never syncytial and there is a distinct cycle of nuclear changes com parable to those seen in the thoracic gland of insects Further reports of histological studies are awaited with great interest The lateral organs described by Silén (1954) in Pericarida may be ventral glands

v The vas deferens gland This structure is the only differentiated en docrine gland among the invertebrates known to be directly involved in the production of a sex hormone It was discovered by Charniaux Cotton in the amphipod *Orchestia gammarella* in 1954, and she has been responsible for nearly all the subsequent work on its structure and function (Charniaux Cotton, 1954a,b, 1956a,b c, 1957, 1958a,b) It is a small gland on the surface

of the vas deferens made up of small cells, many of which are in mitosis The gland has been found in *O gammarella*, in the anomuran *Clibanarius misanthropus*, the natantian *Lysmata seticaudata*, and the brachyuran *Carcinus maenas* The name "androgenic gland," based on its function, was proposed by Charniaux Cotton, but Knowles and Carlisle (1956) suggest the anatomical term "vas deferens gland"

Legrand (1955a, 1958b) was unable to find the vas deferens gland in several isopods, but Balesdent Marquet (1958) has identified it in *Asellus aquaticus* In the other isopods, the function of sex hormone production appears to reside in the nutritive cells of the testis and it appears possible that the vas deferens gland is derived from such cells which have migrated out of the gonad

d Other Arthropods In *Scutigerella immaculata* (Symphyla) Jupeau (1956) has described two pairs of glandular structures which he calls fusiform organs They are located medioventrally in the head, are innervated from the subesophageal ganglion, and have no external duct They contain two kinds of cells, small and large, with secretion granules in the latter They resemble the ventral glands of pterygote insects but differ from the latter, as do the ventral glands of most decapod crustaceans, in that they persist in the adult

In chilopods, Palm (1956) and Gabe (1956a) have added to previous descriptions of the cerebral glands These are located close to the brain, the exact position varying with species They are innervated from neurosecretory cells in the protocerebrum The gland tissue as reported by Palm is syncytial, but Gabe points out that cell boundaries can be distinguished by appropriate techniques This comment may also apply to reports of syncytial glands in other arthropods The gland contains droplets of secretion which appear to originate in the axons, and a phloxinophilic secretion which appears to be intrinsic Palm and Gabe agree with previous investigators in regarding the glands as homologs of the corpora cardiaca Similar cephalic glands have been described by Gabe (1955) in diplopods

The central nervous system of xiphosurans has neurosecretory cells (see Gabe, 1954a), and Waterman and Enami (1954) have described a secretory cycle in the lateral rudimentary eye of *Tachypleus tridentatus* The neuroendocrine system of this group of animals deserves some study in view of the isolated character of the group

3 Conclusions

The studies of the past five years have reinforced and extended to several groups previously neglected the concept that most of the endocrine organs of arthropods are end organs of the neurosecretory system The general term "neurohemal organ" has been proposed for such structures A fundamental pattern now appears to be quite uniform through the whole phylum

The cerebral neurosecretory cells send axons in definite tracts to two pairs of end organs located adjacent to but outside the central nervous system In the insects and arachnids, these end organs appear to be modified ganglia and contain both the axon termini and intrinsic secretory cells of one or more kinds In the crustaceans, the end organs appear to be derived from the neurilemma (sinus gland) or a sense organ (Hanstrom's organ) and the evidence for intrinsic secretory cells is contradictory To some extent in the insects, and much more in the crustaceans it is uncertain whether the neurosecretory product, which can be seen in histological preparations or in living cells to migrate along the axons and in favorable cases to be liberated from the axonal endings, is modified in the end organs before liberation into the blood The relation of the neurosecretory product to the intrinsic secretion of the end organ, if any, is not clear, but it seems probable that some at least, of the secretory products of the system cannot be visualized by the usual histochemical methods There is increasing evidence that there are several distinct types of neurosecretory cells in both insects and crustaceans, and the problem of identifying these and establishing their role in various physiological processes is a challenging one It seems clear that some of these neurosecretory cells will liberate their product into the blood stream or possibly directly in contact with the target organ, without the aid of a differentiated neurohemal organ

In the past five years two new endocrine organs not directly associated with the nervous system were described in arthropods namely, the ventral gland (Y organ) of crustaceans and the vas deferens gland (androgenic gland) of the same group Further details of the anatomy and embryology of the ventral gland should help to confirm the evident homology with the thoracic gland of insects and similar structures in arachnids, and further investigation in the control of sexual characters in other arthropods should determine whether male endocrine tissue is characteristic of the whole phylum or only of the crustaceans

III Physiology of the Neuroendocrine System

In this section I have chosen to consider the role of the neuroendocrine system in specific physiological processes, rather than to examine the functions of the several endocrine structures This procedure seems more profitable since the functions of the various structures have been studied largely in terms of their role in a specific process, such as the control of growth, molting and metamorphosis color change or reproduction

1 The Control of Growth and Development

The growth of arthropods is discontinuous, with a rapid increase in size after each molt, followed by a relatively long period in which there is little or no change in weight Drach (1939) first emphasized the fact that

for crustaceans the period between molts is not a period of constant, normal activity, but rather an orderly cyclical sequence of events leading from one molt to the next Wigglesworth (1954a) has used the same concept in considering insect molts We shall use here the simplified subdivision of the period between molts proposed by Knowles and Carlisle (1956), into postmolt, intermolt, and premolt periods, recognizing that these subdivisions are arbitrary and that each of these periods involves an orderly sequence of events rather than a constant condition

a Insects The major reviews in this field are those of Wigglesworth (1954a) and Novak (1959), and limited aspects of the subject have been covered by Bodenstein (1957, regeneration), Butenandt (1955, 1959, purification and properties of hormones), Hinton (1957, diapause), P Joly (1958, *Locusta migratoria*), Lees (1955, diapause), Schneiderman (1957, diapause), Wigglesworth (1956a, 1957, metamorphosis) The basic pattern of control of molt is now so well known that only a brief summary is needed here The initial endocrine stimulus arises in the pars intercerebralis of the brain This stimulus, presumably through a product of the neurosecretory cells of this region, activates the thoracic gland, this in turn liberates a factor that activates the epidermal cells, inducing mitosis and the subsequent premolt changes of cuticular secretion In larval stages, developmental changes in the epidermis and other tissues during premolt are prevented by a factor originating in the corpora allata When this factor is eliminated by removal of these glands, or when, as in later molts of normal development, the glands fail to secrete, more or less extensive reorganization of tissues occurs in the premolt period and the molt is accompanied by metamorphosis

In general, insects falt into three categories the Ametabola, in which there is no great change in form after hatching, the Hemimetabola or Heterometabola, in which there is a single change in form at the last or imaginal molt, from the larva to a sexually active imago, and the Holometabola in which there are two changes in form, the first from larva to pupa at the penultimate molt, and the second from pupa to imago at the last molt Nearly all experimental work has involved the last two types of insect In Holometabola, the pupal molt and the imaginal molt are determined by the same factors noted above activation of the epidermis by the secretion of the thoracic gland, plus some retardation of development by the secretion from the corpus allatum The corpus allatum hormone is presumably secreted in progressively smaller amounts in larval, pupal, and imaginal molts Pupal diapause apparently results from a decrease in secretory activity of the thoracic gland, the resumption of development has been clearly shown to involve activation of the brain and the consequent activation of the thoracic gland

i *Molt and metamorphosis the brain and end organs* The evidence establishing the role of the neurosecretory system of the brain, and associated end organs in control of molt and metamorphosis consists of (1) correlation of secretory cycles in the pars intercerebralis cells with events in the molt cycle, (2) observations that removal of the neurosecretory system or its products by extirpation or ligation interferes with molting, and (3) proof that restoration of the missing elements by implantation or parabiosis will restore normal function The existing evidence has been supplemented by ligation experiments in coleopterans (Stellwaag Kittler, 1954, Nuñez, 1954) Ligation experiments have implicated the head region in control of the embryonic molt of *Locusta migratoria* and *L pardalina* (Orthoptera) (Jones 1956a) and of *Didacus ciliata* (Diptera) (Narayanan and Lal, 1954), and of the color change preceding pupation in *Cerura vinula* (Lepidoptera) (Buckmann, 1956, 1959) In the latter insect the color change which involves deposition of pigment in epidermis and fat body, and which occurs before the critical period for initiation of pupal molt, is induced by injection of the purified hormone ecdysone (Section IV, 1) which duplicates the effect of the thoracic gland in molting Buckmann concludes that the color change is brought about by the same hormones involved in the control of molting, but acting at a lower threshold Hidaka (1956, 1957, 1959) has also provided evidence that control of the color change which precedes *pupation in lepidopterans resides in the brain*

Rehm (1955) has counseled caution in interpreting studies correlating secretory activity, as shown by the presence in the secretory cells of granules, with physiological events, on the grounds that accumulation of granules in the neurosecretory cells of lepidopteran larvae occurs after, and not before, the period of active secretion Lhoste (1957) reports appearance of secretory product in the corpora cardiaca of the earwig *Forficula auricularia* (Dermaptera) before such product appears in the pars intercerebralis cells, from such observations, it would be unsafe to reach a decision concerning the causal sequence relating secretion in these two structures In a study of secretory processes related to molting in *Carausius morosus* (Orthoptera), however, Herlant Meewis and Paquet (1956) followed the whole sequence of events from appearance of secretion in the neurosecretory cells to its discharge from axon termini, and correlated these phenomena with other evidences of activity such as mitosis in the corpora allata and the thoracic gland They conclude that, during the first half of the intermolt cycle, neurosecretory product is formed in the intercerebralis cells and transported along the cardiac body nerve The intrinsic product of the corpus cardiacum is present in the intercellular spaces of that organ early in this period and is later discharged into the aorta Both the corpora allata and the thoracic gland show active mitosis In the second half of the

intermolt period, there is a second secretory cycle in the intercerebralis cells In this period, the corpora cardiaca form their intrinsic secretion which is retained within the cells, while the corpora allata are engaged in active secretion This type of study, if correlated with experimental studies, should go far in elucidating the causal relations among the various phenomena observed, and with the physiological events of the intermolt cycle

The anatomical relation of the corpora cardiaca to the intercerebralis cells suggests some functional relationship, but the evidence for the latter, as regards molt, is very limited E Thomsen (1952) had shown that implants of corpora cardiaca from the fly *Calliphora erythrocephala* into brainless diapausing lepidopteran pupae activate the thoracic gland and induce metamorphosis Possompés (1958) reports that section of the cardiac body nerve in the same fly prevents molting, though the same operation in other insects has no such effect The anatomical arrangements of the corpora cardiaca in dipterans, where they are part of the ring gland, make experimentation difficult In other insects, where operations on the corpora cardiaca are much easier, no effect on molting can be found Extirpation of the corpora cardiaca from *Carausius morosus* delays, but does not prevent, the molt (L'Hélias, 1956b) Implants of corpora cardiaca from lepidopterans into brainless diapausing lepidopteran pupae do not activate the thoracic gland (Williams 1956b, Ichikawa and Nishitsutsuji Uwo, 1959) One can only conclude, on present evidence, that the corpora cardiaca are not generally involved in the effect of the pars intercerebralis in activating the thoracic gland, though they may be so involved in a few instances, the discharge of intrinsic product from the corpora cardiaca during the first half of intermolt remains without known significance

The experiments of extirpation and implantation of whole brains, or of the pars intercerebralis alone, have been repeated by Monro (1956) in *Phalaenoides glycine* (Lepidoptera), Nayar (1956c) in *Iphita limbata* (Hemiptera), and Kobayashi (1958) and Kobayashi and Kirimura (1958) in *Bombyx mori*, with the classic results The latter authors, however, add the important information that they have prepared and concentrated active extracts of the brain Gilbert and Schneiderman (1959b,c) report that their active preparations of the juvenile hormone (see Section IV,3) will also activate the thoracic gland, and they consider it possible that the brain hormone is similar to or identical with the juvenile hormone, which is secreted by the corpora allata

ii Molt and metamorphosis the thoracic gland The work of Fukuda, Williams Wigglesworth and Bodenstein in establishing by experiments of ligation, parabiosis, and implantation, that the thoracic gland is responsible for initiation of premolt in insects, has been reviewed by Wigglesworth (1954a) Recent studies have not made necessary any major revision of the views he presents, though they have raised some questions of detail

Extirpation of the thoracic gland is difficult in most insects because of the diffuse nature of the gland The operation has been accomplished in the orthopterans *Locusta migratoria* (Deroux Stralla, 1948, Strich Halbwachs, 1954, P Joly *et al*, 1956, P Joly, 1958) and *Periplaneta americana* (Bodenstein, 1953, Chadwick, 1955, 1956) If the gland is removed from *L migratoria* early enough in the fourth or fifth nymphal stages, the animal fails to molt The same operation in *P americana*, however, does not interfere with molt Chadwick suggests that there is some source of the molting hormone out side the thoracic gland, Bodenstein had earlier shown that the thoracic gland of this species contains the molting hormone

In this connection, the experiments of Wigglesworth (1955a c, 1956b) with *Rhodnius prolixus* (Hemiptera) are of special interest He finds that injections of substances such as India ink into the nymphs will delay molt in much the same way as does decapitation The effective substances are those which are taken up massively by the amebocytes The critical period after which molt is not blocked is later than that for decapitation or isola tion of the abdomen, and this suggests a temporal sequence in which the amebocytes are involved The amebocytes have a secretory cycle which reaches its maximum just after the critical period for blocking of these cells, and blocking of the cells decreases the secretory cycle of the thoracic gland Wigglesworth considers that the amebocytes may secrete an activator for the thoracic gland, an alternative possibility is that they secrete a hormone similar to that formed by the thoracic gland Blocking of the amebocytes does not prevent the initiation of premolt which is brought about by injec tion of the hormone ecdysone

The major event in the period under review has been the isolation of ecdysone by Butenandt and Karlson (1954) This hormone duplicates the effects of the thoracic gland in initiating molt and metamorphosis, and its availability in limited quantity in crystalline form, and in larger amounts in purified form, has permitted several investigations of its mode of action The assay used by Karlson (see Section IV,1) depends upon the darkening of the isolated abdomen of larvae of the fly *Calliphora erythrocephala*, as an index of the initiation of pupation Karlson and Schmid (1955) have shown that the action of the hormone is not exerted on the enzyme tyrosinase, which is concerned in the darkening Hanser and Karlson (1957) have been able to initiate pupal development in *Ephestia kuhniella* (Lepidoptera) by injecting relatively large doses of ecdysone development is, however not completed, whether the hormone is administered in a single dose or in sev eral successive doses The hormone induces both epidermal mitosis and cuticle secretion Epidermal mitosis can be induced locally, in absence of the hormone, by burns or other wounds but cuticle secretion does not occur unless the hormone is injected, or the thoracic gland activated These ob servations may be related to those from Joly's laboratory (Halbwachs *et al*,

1957, P Joly, 1958) on extirpation and implantation of thoracic glands in *Locusta migratoria* In the absence of the thoracic gland, epidermal mitoses continue slowly, and eventually reach the level characteristic of premolt, though there is no secretion of cuticle When the thoracic gland is now implanted into animals which have completed epidermal mitoses spontaneously without the thoracic gland, molt is not induced It appears that the thoracic gland hormone, or ecdysone, stimulates but is not essential for epidermal mitosis, and is essential but not sufficient for cuticle secretion Karlson and Buckmann (1956) and Buckmann (1959), as noted earlier, have shown that ecdysone will elicit the color change which accompanies formation of the prepupa in *Cerura vinula* (Lepidoptera)

The hormone of the thoracic gland is often characterized as a "growth and differentiation hormone" Luscher and Karlson (1958) question the applicability of this terminology to ecdysone, on the basis of experiments with termites The metamorphosis and sexual maturation of larvae of these animals is inhibited by an "ectohormone" which is spread through the colony by the larvae Ecdysone elicits premature molts, but not imaginal differentiation or maturation of oocytes, it appears to act simply as a molting hormone Many have assumed that ecdysone is the hormone of the thoracic gland, on the basis of the similarity of the actions of the hormone to those of thoracic gland implants It is important to note, however, that there is no direct evidence for this identity, ecdysone was isolated from the whole bodies of *Bombyx mori* pupae, and its presence in the thoracic gland has not been directly demonstrated In this connection it is significant that Karlson and Stamm Menendez (1956) have found ecdysone in the bodies of *Bombyx mori* imagines, where the thoracic gland has degenerated Such degeneration is general in adult insects (Wigglesworth 1954a, 1955b, Formigoni, 1956), but Carlisle and Ellis (1959) have found the gland persisting in solitary males of *Locusta migratoria*, and in females of *L migratoria* and *Schistocerca gregaria*

iii Molt and metamorphosis the corpora allata The determination of whether a particular molt will or will not involve developmental changes leading to modification of the form of the insect depends in part on the age of the insect or the competence of the tissues to undergo such changes, but to a much greater extent it depends on the balance of factors secreted by the thoracic gland on the one hand, promoting metamorphosis, and by the corpus allatum on the other hand, retarding developmental change (Wigglesworth, 1954a) The corpus allatum factor is generally referred to as the juvenile hormone A considerable amount of attention has been given to the corpus allatum and its product during the last few years Histological studies have been concerned with the relation of neurosecretory activity to secretion in the corpus allatum The volume of the corpus allatum of the

bee *Apis mellifica* increases continually during larval development, decreases sharply at pupation and then increases, becoming very large in workers, smaller in queens, and smallest in drones (Formigoni, 1956) Herlant Meewis and Paquet (1956) consider that the first wave of neurosecretory activity in the intercerebralis cells of *Carausius morosus* (Orthoptera) during the molt cycle coincides with mitosis in the corpora allata, while the second wave corresponds with discharge of secretion by the corpora allata Bounhiol (1957) has cut the nervi corporis allati of *Bombyx mori* larvae This operation is followed by a slight, and probably insignificant decrease in pupation, but a distinct decrease in percentage of emergence as compared with operated controls In the operated animals, the corpora allata contain large amounts of chromophile substance similar to that in the nerve trunk, and this is retained for a considerable period of time These two sets of observations suggest that the neurosecretory stimulus may be needed for the liberation of the neurosecretory material from the corpus allatum Whether this material corresponds to the juvenile hormone is not established Lhoste (1957) notes no cyclical changes in the corpus allatum of *Forficula auricularia* (Dermaptera) before the imaginal molt (fourth) He describes secretory activity in the pericardial cells of the third and fourth molts only and it is noteworthy that Engelmann and Luscher (1956a) found that accidental or deliberate damage to the pericardial cells of *Leucophaea maderae* (Orthoptera) leads to extra nymphal molts and suppression of the mitotic wave in the corpora allata which normally accompanies the molt They conclude that suppression of corpus allatum secretion in the imaginal molt is brought about through mediation of the pericardial cells The pericardial cells involved here are not the same as the pericardial glands of Diptera, which are homologous with the thoracic gland Evidence that the corpora allata of larval *Leucophaea maderae* are inhibited, perhaps by the brain is briefly noted by B Scharrer (1958), who observes that section of the nervi corporis cardiaci is followed by increased activity in the corpora allata

The corpora allata are in many insects, favorably situated for surgical removal and the study of their function by extirpation and transplantation has been actively pursued in recent years Wigglesworth (1954b) has transplanted corpora allata and ring glands from *Calliphora erythrocephala* (Diptera) into *Rhodnius prolixus* (Hemiptera) and shown that both structures have juvenile activity L Joly (1955) has transplanted corpora allata into the last nymphal stage of *Locusta migratoria* and observed an inhibition of the intense cellular multiplication which occurs in the wing buds in the last stages P Joly (1958) suggests that implanted corpora allata stop development in *Locusta migratoria* at the point it had reached at the time of implantation Piepho and Holz (1959) performed experiments involving

transplantation of midgut tissue of *Galleria mellonella* (Lepidoptera) and in this way obtained evidence that the juvenile hormone acts to retain larval characters in the endoderm as well as in the ectoderm Buckmann (1959) considers that the corpora allata inhibit the color change which normally occurs at pupation of *Cerura vinula* Possompès (1957) has removed the corpora allata from *Sipyloidea sipylus* (Orthoptera) and found that the next succeeding molt forms an adultoid which is more closely similar to the adult as the operation is performed in later instars This contrasts with the earlier results in the related *Carausius morosus*, which always molts twice after allactectomy at any stage before reaching the adult condition Possompès' results agree with the earlier studies in suggesting that the ability of tissues to respond to hormonal stimulus changes progressively from instar to instar Lahargue (1957) has implanted imaginal corpora allata of *Bombyx mori* into larvae of the same species and observed inhibition of pupation in 75% of cases in hosts of the fifth instar In earlier instars the implants produce abnormal development or are toxic When the head is ligated, pupation is prevented or delayed in normal larvae Implants of imaginal corpora allata hasten pupation

The endocrine activity of adult corpora allata is well established Lahargue (1959) has implanted imaginal corpora allata into larvae of *Bombyx mori* which had previously been allatectomized and has observed juvenilizing action Williams (1959) has implanted imaginal corpora allata of *Platysamia* (= *Hyalophora*) *cecropia* (Lepidoptera) into brainless diapausing pupae (Section III,1,a,v) and has observed in some cases the termination of diapause and initiation of development with emergence after molt of animals showing a mosaic of pupal and adult characters As Williams had shown earlier, active brain or thoracic gland implants will terminate diapause and produce typical adults The corpora cardiaca alone have no effect in this situation If brains are implanted subsequent to the corpora allata, mosaic development is initiated in all pupae instead of in a small fraction as with corpora allata alone The effect of the brain can be replaced by injection of ecdysone Implants of corpora allata into the isolated abdomen have no effect unless ecdysone or thoracic glands are also added, then a molt occurs with retention of pupal characters Williams concludes that the imaginal corpus allatum forms juvenile hormone and that this hormone will not itself initiate molt The molts observed following implants of corpora allata alone result from activation of the thoracic gland by some product of the corpora allata Ichikawa and Nishitsutsuji Uwo (1959) report the same result, namely that implants of corpora allata into *Philosamia* (= *Samia*) *cynthia* will induce molting in brainless diapausing pupae Pupal corpora allata stimulate molt and permit normal metamorphosis, but glands from young larvae cause a molt in which pupal characters are re

tained They conclude that the factor which initiates molting may not be the same as the juvenile hormone

Gilbert and Schneiderman (1957, 1958b,c) have developed an assay for the juvenile hormone based on its effect in retaining pupal characters in lepidopteran pupae at the imaginal molt They have surveyed fifteen species of adult Lepidoptera and find juvenile hormone in both sexes, with much more in the male than in the female In some species (e g *Platysamia cecropia*) the female has no detectable juvenile hormone and parabiosis experiments demonstrate a mechanism in the female which inactivates or removes the hormone (Gilbert and Schneiderman, 1959a) The hormone is present also in eggs, embryos, early larvae and diapausing pupae of lepidopterans and in coleopterans Remarkably Gilbert and Schneiderman (1958a) find considerable activity in the vertebrate adrenal cortex as well as in crustacean tissue They report that extracts of the abdomens of male *P cecropia* which contain much juvenile hormone activity, will initiate molts in pupae if the thoracic gland is present, but not otherwise Concentration procedures which increase juvenile hormone activity also increase the activity in initiating molt They therefore consider that the factor in the corpora allata which activates the thoracic gland is the juvenile hormone (Gilbert and Schneiderman 1959b c)

iv Molt and metamorphosis conclusions The results of the work of the last five years appear in general to confirm the basic picture of hormonal control of molt and metamorphosis presented by Wigglesworth (1954a) The brain is clearly the site of origin of an activating hormone and the hormone in all probability arises in the neurosecretory cells of the pars intercerebralis but the histological evidence concerning the cells of origin requires further experimental confirmation from extirpation and implantation experiments These may prove extremely difficult in all but a few species The availability of an effective brain extract should be helpful in studying the effects of the activating hormone, but the identity of this hormone with any stainable neurosecretory product cannot be defined as yet The route by which the activating hormone reaches the circulation and the thoracic gland remains uncertain The corpora cardiaca may be involved in some instances but probably are not in others The role of the amebocytes, whether as sources of activating hormone transporters of the hormone or sources of the molt hormone, is not clear The evidence that the corpora allata may activate the thoracic gland raises the possibility that the juvenile hormone itself may be the activating hormone The role of the thoracic gland as the source of the hormone which activates the epidermal and other cells to mitotic activity and stimulates secretion of cuticle by the epidermis is firmly established It seems highly probable that ecdysone is the product of the thoracic gland, but there is some evidence suggesting a

source of molt hormone outside the thoracic gland and no proof that ecdysone actually originates here The corpora allata are evidently a major source of the juvenile hormone and probably the only one The brain appears to exert a stimulating effect on allatum secretion and probably an inhibitory effect as well The pericardial cells may have a role in the inhibition The mechanism of juvenile hormone action remains obscure, as indeed does the mechanism of action of all arthropod hormones, but there is some evidence that the juvenile hormone inhibits mitosis in regions where this process is especially active

v Diapause The physiology of diapause has been reviewed by Lees (1955) and more briefly by Hinton (1957) and Schneiderman (1957) We shall be concerned here only with embryonic diapause, an arrest of development in the embryo before hatching, and with larval or pupal diapause, an arrest of growth and other activity in one of these stages

Embryonic diapause has been studied principally in the commercial silkworm *Bombyx mori* Some races of this species lay eggs which undergo diapause when the mother has been exposed to specific conditions of temperature and photoperiod in larval or early pupal life The factor which determines the egg toward diapause is secreted by the subesophageal ganglion, and the brain may either elicit or inhibit liberation of this factor Hasegawa (1957) has prepared active extracts containing the factor, which he considers to be liberated from the subesophageal ganglion just after pupation Morohoshi (1959), on the basis of transplantation experiments, concludes that there are two factors, one of which originates in the subesophageal ganglion and causes production of diapause eggs, the other of which originates in the corpora allata and causes production of nondiapause eggs The brain controls both secretions, depending on external stimuli Jones (1956a) considers on the basis of histological evidence that the termination of embryonic diapause in *Locusta pardalina* (Orthoptera) is not determined by organized endocrine centers in the embryo

Pupal diapause has been very thoroughly analyzed in cecropia (*Platysamia cecropia*) moths by Williams and collaborators The diapause condition is characterized by a low respiratory rate, increased resistance to cold, and cessation of development If a diapausing pupa is chilled for a time, and then returned to higher temperatures, the respiratory rate increases, epidermal mitosis begins, and development is resumed Chilling activates the brain, and this in turn activates the thoracic gland (Lees, 1955, Wigglesworth, 1954a) In the period covered by this review, Schneiderman and Williams (1954a,b) and Harvey and Williams (1958a,b) have completed a series of papers showing that the first event in initiation of development is the resynthesis of components of the cytochrome system, which are lacking during diapause Harvey and Williams (1958a,b) and Kurland and

Schneiderman (1959) agree that the factor specifically resynthesized is cytochrome c which disappears in diapause

Van der Kloot (1954 1955) and Williams (1956a) find that the onset of diapause is accompanied by cessation of secretory activity in the neuro secretory cells of the brain disappearance of the enzyme cholinesterase from the brain, and cessation of electrical activity When the diapausing pupa is chilled for a period long enough to break diapause and then returned to room temperature, the enzyme reappears, followed by return of electrical and neurosecretory activity During diapause, and especially during chill ing acetylcholine accumulates and then rapidly disappears when the acetyl cholinesterase is reformed Van der Kloot (1954 1955) considers that the accumulation of acetylcholine is the primary cause of resynthesis of the esterase and that formation of the enzyme restores nervous function and thus initiates neurosecretory activity Williams' (1956a) analysis of tem perature effects is consistent with this as is the fact that Monro (1958) has been able to induce an artificial diapause in *Phalaenoides glycine* (Lepi doptera) pupae by injecting eserine (physostigmine), which inhibits acetyl cholinesterase

Highnam (1958a,b) finds that control of diapause in *Mimas tiliae* (Lepi doptera) is generally similar to the control in cecropia, except that the activating factor leaves the brain of *M tiliae* during a relatively short period by comparison with cecropia Highnam considers that termination of diapause is a consequence of a change in the balance of brain and corpus allatum factors rather than an effect of the brain hormone alone Schneider man (1955), Schneiderman *et al* (1956), and Schneiderman and Horwitz (1958) have studied temperature relations in initiation and termination of larval diapause in the wasps *Mormoniella vitripennis* and *Tritneptis klugii*, and have analyzed these effects theoretically in terms of competing proc esses with rates differentially affected by temperature, the specific proc esses have not been identified, but could well be similar to those described in cecropia by Van der Kloot Church (1955) and Fraser (1957) have correlated neurosecretory processes with larval diapause of the sawfly *Cephus cinctus* and the fly *Lucilia caesar*, respectively they find that both medial and lateral protocerebral neurosecretory cell groups show cyclical changes associated with onset and termination of diapause

Wigglesworth (1954a) suggests that diapause is essentially a cessation of growth, and Lees (1955) concurs Smith and Schneiderman (1954) find that wounds in diapausing cecropia pupae are healed only by cell migra tion, whereas in active insects healing involves epidermal mitosis, the differ ence is attributed to the absence of thoracic gland activity in diapause The phenomenon of diapause offers many advantages for the study of hormone effects and the activation of specific processes by hormones in

that, unlike most normal molts, diapause can be terminated or in some cases induced by specific experimental procedures The problem which is raised by the studies discussed above is whether the results of such studies can be extended to other aspects of hormonal control in insects If the fundamental action of the thoracic gland is to cause synthesis of cytochrome c, then this should be detectable in the activation of the epidermis in normal molts, no evidence for such an action has been obtained If the activation of neurosecretory processes in the brain depends on the synthesis of acetylcholine and cholinesterase, this too should be detectable elsewhere To the present writer, it seems unlikely that the phenomena observed in termination of diapause in cecropia can be generalized to the control of molting, or even the control of diapause in other insects

vi Regeneration Students of growth have always found the process of regeneration a valuable source of information In arthropods regeneration is complicated by the requirements of the intermolt cycle and its study should thus provide valuable information about the control of the cycle as well as about the control of growth

A series of recent studies with *Blatella germanica* (Orthoptera) (O Farrell and Stock, 1953, 1954, O'Farrell *et al*, 1956, Stock and O'Farrell, 1954) has involved experiments of regeneration of single legs and of two legs of a pair when the legs were removed in various stages of the intermolt cycle The results indicate that there is a critical period in the intermolt cycle coinciding with a burst of mitotic activity in the thoracic gland If legs are removed before this time, complete regeneration occurs and a new intermolt cycle is established so that the succeeding molt is delayed by the amount of time elapsing between the last molt and the amputation Evidently the regeneration process diverts the thoracic gland hormone from its normal function of initiating a molt If legs are removed after the critical period, only a minimal growth occurs in the stump and full regeneration occurs during the next intermolt cycle Bodenstein (1955) performed similar experiments with *Periplaneta americana* (Orthoptera) with similar results though with less evidence of a sharply defined critical period He was able to induce regeneration in adult limbs by parabiosis with a nymph, or by transplanting the limb stump to a nymph Removal of thoracic glands did not prevent molt or regeneration Regeneration of nymphal limbs is delayed by parabiotic union with an adult Bodenstein (1957) considers that the hormonal threshold for growth is lower in younger tissues and that the threshold for differentiation is higher than that for growth It seems clear from regeneration studies that the system which activates epidermal mitosis is also essential for regenerative growth

Another aspect of growth which may offer new sources of information is the formation of tumors Certain genetic strains of *Drosophila melanogaster*

(Diptera) have a high incidence of tumors Burdette (1954a,b) found that ligation of the larvae of such a strain posterior to the ring gland late in the third instar, an operation which delays metamorphosis, also increases the tumor incidence The same effect is brought about by introduction of a mutant gene that causes a defect in the ring gland and thus prevents metamorphosis These observations would bear a more detailed experimental analysis to determine if possible what specific hormonal factors are involved

b Crustaceans Studies of the control of growth and development in crustaceans have been much more limited than in the insects This is in part because the common decapods at least offer less favorable material for operative manipulations than do insects and also present less variety in life history Removal of the eyestalks is the standard operative procedure and considerable information has been obtained from this operation but the role of the central nervous system and of endocrine structures in the cephalothorax in the control of molting has been examined only in recent years No studies have been carried out on larval development and the discussion to follow is based on postlarval molts only, which are characteristic of all crustaceans but only of the most primitive insects

i Molt Most of the work on the control of molting in crustaceans has concentrated on the molt inhibiting hormone of the eyestalk Until 1952, it was generally considered that this factor originates in the sinus gland However in 1952 three groups of investigators independently presented evidence that the hormone arises in neurosecretory cells in the medulla terminalis with axon termini in the sinus gland The most extensive physiological study supporting this view is that of Passano (1954b), who showed that removal of the neurosecretory cells of the medulla terminalis (which he calls the X organ) leads to initiation of molting but that removal of other parts of the eyestalk, including the sinus gland will not initiate molt if the terminalis cells are intact Passano (1954a,b) also provided histological and physiological evidence of axonal transport from the terminalis cells to the sinus gland including the observation of such transport in living preparations The specific cells of origin of the molt inhibiting hormone have not been identified Durand (1956) and Matsumoto (1958) have observed secretory changes in specific cells of the medulla terminalis and of the sensory papilla organ respectively, correlated with events of the intermolt cycle, and Rehm (1959) has described changes in staining properties of axon termini in the sinus gland Without correlated physiological observations however it is not possible to assess the significance of these phenomena with any confidence

Carlisle (Carlisle and Dohrn, 1953, Carlisle, 1953a b 1956, 1959a) has published evidence indicating the presence of a molt accelerating hormone in the eyestalks and brain of several crustacean species Some of his work

has been criticized by Drach (1955) and Kleinholz (1957) on the grounds of faulty experimental design, but to the present author there remains a body of evidence sufficient to support the view that a factor in the central nervous system accelerates processes leading to the molt and that this factor is normally active in certain species at least Its possible relation to the brain hormone which activates the thoracic gland in insects deserves attention

The discovery by Gabe (1953, 1956b) of the ventral gland (Y-organ, molt gland) of crustaceans and the apparent homology with the thoracic gland of insects led Echalier (1954, 1955) to examine the role of these structures in molting in crustaceans Bilateral removal of the ventral glands from young *Carcinus maenas* results in cessation of molting Operated crabs were kept alive without molting for one year while controls molted several times Implantation of ventral glands into the operated animals then elicited molts If the glands are removed in the intermolt or very early premolt (D_0) stages, the cycle is interrupted in early premolt (D_1) when the cuticle is in process of formation Tchernigovtzeff (1959) has shown that epidermal mitoses occur in *Leander serratus* only in D_0 and early D_1, and Drach (1944) in the same species had shown that eyestalk removal is effective in shortening the period between molts if performed at any time before early D_1 Jyssum and Passano (1957) have shown that removal of ventral glands prevents molt in crabs of the genus *Sesarma* There is no direct evidence that the ventral gland acts directly on mitosis, but Tchernigovtzeff (1959) has shown that removal of eyestalks increases the intensity and duration of mitotic activity in the epidermis of *L serratus*, and as we shall see, Carlisle (1957b) has provided evidence that the molt inhibiting hormone inhibits the ventral gland in *Carcinus maenas* Durand (1960) has correlated histological changes in the terminalis cells of the crayfish with premolt changes in the ventral gland, and thus identified the possible source of the hormone which activates the ventral glands

Carlisle (1957b, Knowles and Carlisle, 1956, Carlisle and Knowles 1959) has given considerable attention to the role of hormonal factors in the normal life history and intermolt cycles of crustaceans He distinguishes two types of cycle, in forms which molt seasonally, there is in winter a prolonged intermolt period lasting several months, a condition which Carlisle calls anecdysis In summer, the intermolt period is relatively short, and is called diecdysis Most higher crustaceans, after the last larval molt and metamorphosis, undergo a number of juvenile molts before sexual maturity In most forms (e g *Carcinus maenas*), molting and growth continue for some time after the puberal molt in which sexual maturity appears In a few species (e g *Maia squinado*), the puberal molt is the last, and no further growth occurs Carlisle (1959a) has shown that two populations of the prawn *Leander serratus* on opposite sides of the English Channel differ in type of

intermolt cycle The Roscoff population, first studied by Drach (1944), has the anecdysis type of cycle, and eyestalk removal shortens the intermolt period, *the Plymouth population* (Carlisle, 1954c) has the diecdysis type of cycle, and eyestalk removal does not shorten the intermolt period Scheer and Scheer (1954a) found the diecdysis type of cycle in *L serratus* at Naples and Scheer (1960) found the same type of cycle in *L xiphias* at Villefranche sur Mer In the anecdysis type of cycle, the molt inhibiting hormone is presumably secreted during the intermolt, whereas in the diecdysis cycle it is not (Carlisle 1959a)

Carlisle (1957b) has compared the terminal anecdysis of *Carcinus maenas,* which occurs some time after sexual maturity when the animal has attained its maximum size with that in *Maia squinado,* which develops at the time of the puberal molt In *C maenas,* the terminal anecdysis depends on hyperactivity of the molt inhibiting hormone, and additional molts can be induced by eyestalk removal or by implants or *extracts from the* ventral glands of younger animals In *M squinado,* there is little molt inhibiting activity in the sinus gland, and the ventral glands are atrophied, Carlisle considers the latter phenomenon to be responsible for the terminal anecdysis, as is the case in insects where the thoracic glands atrophy at metamorphosis Vernet Cornubert (1959a) finds a similar situation in *Pisa tetraodon,* where removal of eyestalks will not induce molt in puberal forms

Scheer and Scheer (1954a) noted a difference in color of L serratus correlated with differences in intermolt cycle stage Analysis of this difference showed that each of the independent pigment types of the chromatophores of this species undergoes cyclic variation in degree of dispersion, especially during premolt and postmolt stages Scheer (1960) has recently observed a similar cycle in *L xiphias,* and Chassard (1958) finds that the extent of adaptive color change when *L squilla* is placed on a white background varies with intermolt cycle stage Scheer and Scheer (1954a) interpreted their observations as suggesting that the chromatophorotropic hormones have specific roles connected with events in the intermolt cycle and as evidence for this used observations that the duration of specific stages was in several instances correlated with the degree of dispersion of specific pigments in individual prawns

From this discussion it appears that our knowledge of the control of molting in crustaceans is well behind that for insects The only clear analogy between the two groups is in the ventral and thoracic glands, which have the same general effect on molting However further evidence of their exact *role in initiating* premolt is needed Karlson (1956a) has isolated from the prawn *Crangon vulgaris* a substance similar in properties to the ecdysone of insects, suggesting that the chemical basis for initiation of molting is essentially similar in the two groups Karlson and Skinner (1960)

have examined the distribution of ecdysone in the body of *Carcinus maenas*, and find more than 1000 times as much hormone in the body minus ventral glands as is found in the ventral glands themselves This strongly suggests that ecdysone does not originate in the ventral glands The molt inhibiting hormone of crustaceans has no analog as yet in insects and the evidence for a hormone which activates the ventral glands of crustaceans is still very limited Crustaceans evidently form juvenile hormone (Schneiderman and Gilbert, 1958) and the eyestalks are the richest source, but the role of this hormone in development remains unexplored since no studies have been made of the control of larval molts

ii Regeneration The relation of regeneration to molting has been studied in the land crab *Gecarcinus lateralis* by Bliss (1954a,b, 1956) and in crayfish by Durand (1960) Limb regeneration appears to follow much the same pattern described in insects Multiple amputations will initiate a molt under conditions in which this would not ordinarily occur Molting and limb regeneration in the land crab are dependent for their initiation on environmental factors, which probably exert their actions through humoral factors in the eyestalks Echalier (1956) has found that regeneration does not occur after removal of the ventral glands from *Carcinus* (= *Carcinides*) *maenas*, and Jyssum and Passano (1957) report only basal growth in a species of *Sesarma* after this operation The limited evidence suggests that in crustaceans as in insects regeneration is under the same control as is initiation of premolt

c Arachnids The extensive histological work of the last few years on the arachnids has not yet led to any physiological studies However, Herlant Meewis and Naisse (1957) and Naisse (1959), in several species of phalangids, have described cycles of secretion in the neurosecretory system and in the thoracic gland correlated with molting The thoracic gland shows mitoses from the first third to the middle of the intermolt cycle, after which a phloxinophilic secretion appears in the cells, and granules with the same staining properties can be seen in the adjacent blood sinus In the last third of the intermolt cycle the secretion disappears The gland regresses after the imaginal molt The secretion in the neurosecretory cells accumulates at the time of molt, and the activation of the thoracic gland coincides with discharge of this secretion from the paraganglionic plaques, which are regarded as homologs of the corpora cardiaca Naisse (1959) has also described a secretory cycle in the blood cells similar to that reported by Wigglesworth (1955a,c, 1956b) for *Rhodnius prolixus*

d Conclusions The basic picture of the control of growth and development in insects as presented at the beginning of this section has not been greatly modified during the last five years It has become clear that regeneration and larval and pupal diapause are under the same basic hormonal

control as is the initiation of premolt The isolation of ecdysone as a crystal line hormone is a considerable advance and offers opportunities for closer studies of the activation process The major problems which have been raised have been those of the exact nature of the relations between the neurosecretory system and the thoracic glands and between the elements of neurosecretory system, the origin of the hormonal factors, and their re lation to the histologically demonstrable neurosecretory products In decapod crustaceans we can now feel more confident that the control of postlarval molting is basically similar to that in insects, but extensive comparative study will be needed to establish the extent of this similarity In other arthropods we know essentially nothing of the control of growth and development, and even in decapods we know nothing of the control of larval molts

2 *The Control of Reproduction*

In contrast with the rather abundant earlier work on growth and develop ment, studies of neuroendocrine control of reproduction have been relatively few until recently Various aspects are covered in the reviews of Carlisle and Knowles (1959 crustaceans) Charniaux Cotton (1958b, crus taceans) P Joly (1958 insects) Kleinholz (1957 crustaceans), Knowles and Carlisle (1956, crustaceans), Novak (1959, insects), Reinhard (1956, crustaceans), B Scharrer (1954 neurosecretion), E Scharrer and B Schar rer (1954, neurosecretion) The situation at the beginning of the period covered by the present review was relatively simple In insects it had been demonstrated that the pars intercerebralis of the brain is necessary for egg development in *Calliphora erythrocephala* The corpora allata were also known to be essential The cytological maturation of the egg can continue normally in the absence of either structure but the deposition of yolk fails to occur It had accordingly been suggested that the hormone involved is not truly a gonadotropic hormone, but a metabolic hormone concerned in mobilization of materials for, among other things, yolk deposition In crus taceans there was evidence that the brain forms a hormone which inhibits ovarian growth and possibly in some cases one which inhibits the testes In insects there was no reliable evidence for sex hormones formed by the gonads and influencing secondary sex characters, and in crustaceans the evidence was limited to demonstration that ovariectomy prevents appearance of the cyclical breeding characters in the female, and numerous observations of changes in secondary sex characters following parasitic castration

a Insects i Egg development The relation of the pars intercerebralis and the corpora allata to egg production has been intensively explored in certain insects during the last several years The development of eggs in adult female insects of certain species is inhibited by specific conditions

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a Insects i Egg development The relation of the pars intercerebralis and the corpora allata to egg production has been intensively explored in certain insects during the last several years The development of eggs in adult female insects of certain species is inhibited by specific conditions

have examined the distribution of ecdysone in the body of *Carcinus maenas*, and find more than 1000 times as much hormone in the body minus ventral glands as is found in the ventral glands themselves This strongly suggests that ecdysone does not originate in the ventral glands The molt inhibiting hormone of crustaceans has no analog as yet in insects and the evidence for a hormone which activates the ventral glands of crustaceans is still very limited Crustaceans evidently form juvenile hormone (Schneiderman and Gilbert, 1958) and the eyestalks are the richest source, but the role of this hormone in development remains unexplored since no studies have been made of the control of larval molts

ii Regeneration The relation of regeneration to molting has been studied in the land crab *Gecarcinus lateralis* by Bliss (1954a,b, 1956) and in cray fish by Durand (1960) Limb regeneration appears to follow much the same pattern described in insects Multiple amputations will initiate a molt under conditions in which this would not ordinarily occur Molting and limb regeneration in the land crab are dependent for their initiation on environmental factors, which probably exert their actions through humoral factors in the eyestalks Echalier (1956) has found that regeneration does not occur after removal of the ventral glands from *Carcinus* (= *Carcinides*) *maenas*, and Jyssum and Passano (1957) report only basal growth in a species of *Sesarma* after this operation The limited evidence suggests that in crustaceans as in insects regeneration is under the same control as is initiation of premolt

c Arachnids The extensive histological work of the last few years on the arachnids has not yet led to any physiological studies However, Herlant Meewis and Naisse (1957) and Naisse (1959), in several species of phalangids, have described cycles of secretion in the neurosecretory system and in the thoracic gland correlated with molting The thoracic gland shows mitoses from the first third to the middle of the intermolt cycle, after which a phloxinophilic secretion appears in the cells and granules with the same staining properties can be seen in the adjacent blood sinus In the last third of the intermolt cycle the secretion disappears The gland regresses after the imaginal molt The secretion in the neurosecretory cells accumulates at the time of molt, and the activation of the thoracic gland coincides with discharge of this secretion from the paraganglionic plaques, which are regarded as homologs of the corpora cardiaca Naisse (1959) has also described a secretory cycle in the blood cells similar to that reported by Wigglesworth (1955a,c, 1956b) for *Rhodnius prolixus*

d Conclusions The basic picture of the control of growth and development in insects as presented at the beginning of this section has not been greatly modified during the last five years It has become clear that regeneration and larval and pupal diapause are under the same basic hormonal

control as is the initiation of premolt The isolation of ecdysone as a crystalline hormone is a considerable advance and offers opportunities for closer studies of the activation process The major problems which have been raised have been those of the exact nature of the relations between the neurosecretory system and the thoracic glands and between the elements of neurosecretory system, the origin of the hormonal factors, and their relation to the histologically demonstrable neurosecretory products In decapod crustaceans we can now feel more confident that the control of postlarval molting is basically similar to that in insects, but extensive comparative study will be needed to establish the extent of this similarity In other arthropods we know essentially nothing of the control of growth and development and even in decapods we know nothing of the control of larval molts

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a Insects *i Egg development* The relation of the pars intercerebralis and the corpora allata to egg production has been intensively explored in certain insects during the last several years The development of eggs in adult female insects of certain species is inhibited by specific conditions

such as lack of food, or of food of a certain sort, or in viviparous insects during pregnancy This inhibition is sometimes, perhaps inappropriately, called a diapause In *Leucophaea maderae* (Orthoptera) and *Oncopeltus fasciatus* (Heteroptera) fasting causes inhibition of egg development (Johansson, 1954, 1955, 1958a,b) In *Culex pipiens pipiens*, *Aedes aegypti*, and *Anopheles stephensi* (Diptera), egg development is anautogenous, it will occur only if the females feed on blood, and not when they feed on fruits In *Culex pipiens molestus*, egg development is autogenous, and will occur even if the insects feed entirely on fruit, egg development in this form, however, can be inhibited by ligation between the head and abdomen, decapitation, or ligation between thorax and abdomen, if the operation is performed soon after emergence of the female (Clements, 1956, Larsen, 1958, Larsen and Bodenstein, 1959) Females of *Leucophaea maderae* also fail to develop eggs if mating is prevented by separating them from males immediately on emergence (Engelmann, 1958, 1959b) Egg development in this viviparous species is also inhibited during pregnancy (Engelmann, 1957)

The inhibition of egg development during fasting in *Oncopeltus fasciatus* appears to originate in the pars intercerebralis, extirpation of the whole brain or of the pars intercerebralis alone will elicit egg development in fasting females (Johansson, 1954, 1958a,b,c) Removal of the whole brain or of the lateral lobes of the protocerebrum from fasting *Leucophaea maderae* will elicit egg development, but removal of the pars intercerebralis alone has no effect The inhibitory effect in this species seems to travel in the nervi corporis cardiaci I and the nervi corporis allati to the corpora allata, since section of either pair of nerves is followed by activation of the corpora allata and egg development in fasting or virgin females, section of the nervi corporis cardiaci II has no effect (Engelmann, 1957, 1958, 1959b) The inhibition during pregnancy of *L maderae* is apparently due to a substance formed by the mature egg and embryo, since presence of eggs or embryos in the body cavity of the female leads to inhibition of egg development (Engelmann, 1957)

The role of the corpora allata as sources of the activating effect for egg development is well established Implants of corpora allata from late last stage larvae or fed adult females of *L maderae* or *C fasciatus* into starved adult females of the same species will activate egg development (Johansson, 1955, 1958a,b,c) Implants of corpora allata from autogenous mosquitoes (*Culex pipiens molestus*) into anautogenous forms (*C p pipiens*) will elicit egg development in the host (Larsen and Bodenstein, 1959) The activating effect of blood feeding in mosquitoes is clearly mediated humorally An autogenous forms such as *Aëdes aegypti* will mature eggs only if fed blood Ligation of the head or decapitation soon after feeding prevents egg de

velopment, but injection of hemolymph from females fed blood into the abdomens of decapitated females will induce egg development in the latter (Gillett 1957, 1958) If larval ovaries from anautogenous *Culex pipiens pipiens* are transplanted into autogenous *C p molestus* females, the implants develop eggs, if ovaries from autogenous females are transplanted into anautogenous females, the implants develop eggs only if the host feeds on blood The critical period during which ligation of the head of mosquitoes prevents egg development coincides with the period after emergence (in autogenous forms) or blood feeding (in anautogenous forms) in which no signs of activation of the corpora allata are evident The period during which ligation of the thorax prevents egg development coincides with the period during which the corpora allata are developing rapidly (Larsen and Bodenstein, 1959) The corpora allata of starved or virgin females of *Leucophaca maderae* remain small Feeding of mated females soon after emergence is followed by an increase in volume and cell number in the corpora allata After a prolonged fast there is an increase in volume without cell multiplication (Scharrer and von Harnack 1958, von Harnack, 1958a,b)

It is not clear to what extent the animals studied so far are typical and whether corpus allatum activity is always normally involved in egg development Possompés (1955 1956) states that removal of the corpora allata from *Calliphora erythrocephala* (Diptera) or *Sypiloides sipylus* (Orthoptera) does not prevent egg development but E Thomsen had earlier shown an interference of this operation with egg development in *C erythrocephala* The discrepancy remains unexplained

The normal stimulus to egg development in *Leucophaea maderae* and *Diploptera punctata* is mating, which occurs soon after emergence In virgin females in which egg development has been elicited by section of the nervi corporis allati there are two cycles of egg development as there are in normal mated females the second cycle follows parturition, and is accompanied by a second cycle of growth of the corpora allata If the egg cases are removed through the vagina before parturition, the second cycle of egg development occurs mechanical stimuli to the external genitalia of virgin females will also elicit corpus allatum growth and egg development Engelmann (1958, 1959b) concludes that afferent stimuli from the genital region, which may arise from mating or parturition, activate the corpora allata The activation can be prevented by section of the ventral ganglion chain or by complete denervation of the corpora allata, this suggests that a direct activation via the subesophageal ganglion is involved, and not merely the release of inhibition from the brain

In *Oncopeltus fasciatus* and the anautogenous mosquitoes, the stimulus to egg development is feeding the effective stimulus is distention of the midgut not the composition of the food (Johansson, 1958a b, Larsen and

Bodenstein, 1959) In the mosquitoes, filling the midgut with milk which like blood, is slowly absorbed, or ligation of the anus while feeding fruit, will elicit egg development Both procedures cause prolonged distention of the midgut Injection of blood into the hemolymph, on the other hand, is ineffective

The pattern of control suggested by these studies is that egg development is normally elicited by the secretion of the corpora allata, the fact that late larval corpora allata are active suggests that the effective agent may not be the juvenile hormone The corpora allata can be inhibited by neuro secretory cells in the brain, the specific region involved seems to be different in the two cases studied, but the pathway of transmission to the corpora allata is the brain allatum tract The inhibition can be released, or the corpora allata activated directly, by stimuli in various body regions such as the midgut or the external genitalia, the direct activation pathway seems to be through the ventral nerve cord and the subesophageal ganglion

Engelmann and Luscher (1957) and Engelmann (1959a) have asked why egg development is not activated during the last larval stage, when the corpora allata are active They find that larval ovaries of *Leucophaea maderae* are competent to develop eggs, and will do so if implanted into adult females Ovaries from young females, however, will not develop eggs if transplanted to larvae The difference is largely attributed to the thoracic gland, which degenerates in the adult Implants of thoracic glands or in jections of ecdysone into young females retard egg development Implants of subesophageal ganglia antagonize the action of the thoracic gland Engel mann considers, however, that the action of the thoracic gland is not suf ficient by itself to account for failure of egg development in larvae, and Strich Halbwachs (1958) finds that removal of the thoracic gland from *Locusta migratoria* before the imaginal molt does not speed egg develop ment It seems probable that a specific stimulus may be required to elicit corpus allatum development, and that the agent effective on egg develop ment may not be the juvenile hormone

Luscher and Engelmann (1955) and Engelmann and Luscher (1956a,b) consider that the nervous pathways involved in inhibition of the corpora allata with respect to their action in egg development are the same as those involved in inhibition in metamorphosis Too little is currently known about the latter phenomenon to assess this suggestion adequately, but it offers an interesting approach to further work E Thomsen and Møller (1959) have removed the medial protocerebral neurosecretory cells from *Calli phora erythrocephala* and observed failure of vitellogenesis suggesting that normally these cells may be responsible for activating this process through the corpora allata in this species at least Johansson's (1958a,b,c) observa tion that extirpation of the "A" cells of the pars intercerebralis of *Onco*

peltus fasciatus slows growth of the corpora allata and egg development in females leads to a similar conclusion

ii Other effects In general, attempts to demonstrate the existence of sex hormones by extirpation or implantation of gonads or other tissues have been unsuccessful in insects Poggendorf (1956) in the apterygote *Orchesella cincta* (Collembola) reports a correlation between the presence of a male secondary sex character and the presence of spermatophores, but he has no evidence of a humoral link between these Newcomer (1955) found no change in the glycogen content of muscles of male cockroaches (*Periplaneta americana*) after castration, although the male has about three times as much muscle glycogen as the female Johansson (1958b) castrated both sexes of *Oncopeltus fasciatus* (Heteroptera) and observed no change in secondary sexual characters, volume of corpus allatum, or neurosecretory cells The female fat body hypertrophied, but this same effect could be produced be removal of the corpora allata and consequent failure of egg development Removal of the accessory sex glands was also without effect

The development of the sexually mature queen bee from larvae which can become either queens or workers appears to involve endocrine factors Schaller (1955) has shown that the thoracic gland of queens is much larger than that of workers or drones, and Lukoschus (1955a, 1956b) reports that the corpus allatum is much larger in larvae destined to be queens than in those which will become workers During pupation there is a decrease in size of the gland in queens Formigoni (1956) has observed similar changes Lukoschus (1955b 1956a c d) has studied the development of the distinguishing features of queens and workers He finds an increased epidermal cell division to be basically responsible for the difference In his view the queen is normal and comparable to sexually mature forms of other insects, while the development of workers is inhibited by a hormonal factor secreted in response to nutritional deficiency Experimental studies are lacking

The well known fact that there is only one queen in a hive depends on the secretion by the queen of an ovary inhibiting substance which is spread through the hive by contact (De Groot and Voogd, 1954, Pain 1954) The secretion of the queen includes in addition a substance which is attractive to workers and an antibiotic These are distinct from the hormone (Lavie and Pain 1959) The hormone has been purified by Butler *et al* (1959), and seems to be an unsaturated aliphatic acid

In termites *Anoplotermes pacificus*, the larvae which will develop sexually have larger thoracic glands than do those which will become workers (Kaiser 1955) An ectohormone acts to inhibit sexual development in *Kalotermes flavicollis* colonies as in bees Removal of sexual forms from the colony is followed by a molt in certain larvae or nymphs which transforms them into neotenic sexual forms These animals and normal sexual forms

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have a group of active neurosecretory cells in the brain not present in larvae and present but inactive in pupae (Noirot, 1957) Luscher (1956, 1957, 1958) has extracted from the heads of the supplementary sexual forms a substance which stimulates development of all castes Activation of the thoracic glands and corpora allata occurs at the time of determination of the supplementary sexual forms The metamorphosis of these forms does not involve inactivation of the corpora allata as does normal metamorphosis, but it is followed by degeneration of the thoracic glands Luscher (1957) suggests that this degeneration is normally caused by sexual maturation and not by absence of secretion from the corpora allata Implantation of corpora allata into larvae long before molt and before activation of the thoracic glands causes the larvae to develop into soldiers

b Crustaceans In insects, the development of the definitive adult form in the final molt is accompanied by sexual maturation In most of the crustaceans commonly studied (decapods, isopods), this is not the case, and the last larval molt is followed by several juvenile molts, in which the animal, though adult in body form, remains sexually immature Development of the gonads occurs at the time of a puberal molt, and external secondary sexual characters also generally appear at this time Some of the latter may be cyclical characters, in that they appear at the molt which precedes the breeding season and disappear at the following molt, when the reproductive activity is ended for the year Some decapods exhibit consecutive protandric sexuality, the puberal molt is accompanied by maturation of the gonads as testes in all individuals and development of male secondary characters At a later molt, sex reversal occurs, the gonad is converted to an ovary, and female characters appear The control of these phenomena involve gonadotropins formed in the neurosecretory cells of the central nervous system, and sex hormones formed in the gonads

i Gonadotropins Evidence pointing to the existence of an ovary inhibiting hormone in the eyestalk was first obtained by Panouse (1943), who showed that eyestalk removal from immature female crabs is followed by rapid ovarian development This experiment has been repeated in many species with similar results The Nancy group has shown, during the period covered by this review, that eyestalk removal from prepuberal female crabs (*Carcinus maenas*) induces a molt and ovarian development, but the molt is not a typical puberal molt in that it is not accompanied by appearance of secondary female characters, ovarian maturation and even repeated oviposition may occur in eyestalkless females in the absence of the structures essential for normal attachment of the eggs to the female abdomen (Cornubert, 1954, Demeusy and Lenel, 1954, Vernet Cornubert and Demeusy, 1955, Demeusy 1958) The source of the ovary inhibiting hormone has been established, in the prawns *Lysmata seticaudata* and *Pandalus borealis*,

as the medulla terminalis sinus gland system (Carlisle, 1954a, 1959d) In *P borealis*, which is a protandric hermaphrodite, ovarian development is prevented in the male phase by the ovary inhibiting hormone, since removal of the eyestalks or the terminalis sinus gland neurosecretory tract initiates transformation of the ovary into a testis, while extracts of these regions injected into males in which testicular degeneration has begun retards transformation of the testis into an ovary (Carlisle, 1957a, 1959b,c,d) Eyestalk removal accelerates vitellogenesis in mature females of *Leander serratus* (Drach, 1955) The ovary inhibiting hormone appears to have no effect on testicular development in *Lysmata seticaudata* (Carlisle, 1954b), but eyestalk removal from immature male *Carcinus maenas* provokes maturation of testes (Demeusy, 1958) Carlisle and Butler (1956) suggest that the ovary inhibiting hormone of crustaceans is related to the "queen substance" of the bee Extracts of mated queen bees inhibit ovarian development in eyestalkless females of *Leander serratus* and extracts of the sinus gland of *L serratus* fed to newly emerged bees will inhibit ovarian development

The observation has often been made that molting is retarded in gravid female crustaceans, Crisp and Patel (1958) have added barnacles to the list of species for which this is true, but Scheer (1960) could not find any retardation in three natantian species Cornubert (1954) removed eyestalks from gravid females of *Carcinus maenas* and thus induced a molt in which the embryos were lost, but Scheer (1960) found no such effect in *Leander xiphias* and Drach (1955) observed no shortening of the intermolt following oviposition in *L serratus*, although eyestalk removal normally shortened the intermolt in nongravid animals It seems likely that there will be considerable variation in the occurrence of retardation of molt in gravid females from species to species, but in those species where the phenomenon occurs, study of the mechanism should be rewarding

The relation of the ventral gland to ovarian development is, at the moment, obscure Arvy *et al* (1954, 1956) removed this structure from immature crabs (*C maenas*), and observed cessation of gametogenesis and degeneration of the gonads, no such changes followed the operation in mature crabs Demeusy (1959), using the same species, found no degeneration of the gonads after removal of the ventral glands, or of these organs and the eyestalks as well but rather a stimulation of testicular growth in males and of vitellogenesis in females From the accounts available, it is not possible to reconcile the difference in results

Secretory activity in specific neurosecretory cells can be correlated with ovarian development, ovulation, and gravidity in crabs (Parameswaran, 1955, Matsumoto 1958) Matsumoto has identified a specific group of cells in the medulla terminalis, with axons running to the sinus gland which

he thinks are the source of the ovary inhibiting hormone The "onion bodies" of the sensory papilla organ of *Pandalus borealis* show cyclical activity correlated with the reversal of sex in this animal (Carlisle, 1959b) The axons with which these structures are associated arise in the medulla terminalis, but the cells concerned are distinct from the terminalis cells which innervate the sinus glands This observation suggests that a neurosecretory factor other than the ovary inhibiting hormone is also concerned in the reversal of sex in this prawn

ii Sex hormones Attempts to demonstrate the existence of sex hormones in crustaceans as in insects have involved experiments of extirpation and transplantation of gonads These have not in general been successful except in a few instances to be noted later and attempts have been made to destroy the gonads by irradiation In the period covered by this review Balesdent Marquet (1954, 1955a,b) and Balesdent Marquet and Veillet (1958a, 1959) irradiated ovaries in the isopod *Asellus aquaticus* and observed that the incubating pouch characteristic of mature females failed to appear Irradiation of the head regions suppressed this same character but did not stop oviposition

The effects of parasitic castration have also often been cited as evidence for sex hormones in crustaceans This literature has been reviewed by Reinhard (1956), and he concludes that the modifications in secondary sex characters of crustaceans induced by parasites which either destroy or inhibit development of the gonads must involve a disturbance in the balance of genetically determined sex hormones Veillet (1955) considers it more likely that the primary effect of the parasite is on the metabolism Vernet-Cornubert (1958, 1959a,b) finds that the changes in the pleopods of *Pachygrapsus marmoratus* induced by *Sacculina carcini* are not reversed on death of the parasite or after eyestalk removal Eyestalk removal after infection but before initiation of changes of form will retard these changes, especially in the male The differences are attributed to metabolic effects More recently, Veillet and Graf (1959) have observed degeneration of the vas deferens gland in parasitized male crabs, and conclude that this degeneration is induced by the parasite and causes the feminization

Successful transplantation, between sexes, of ovaries and testes was accomplished by Charniaux Cotton (1954a) in the amphipod *Orchestia gammarella* Testes implanted into females were not altered in structure and had no effect on secondary sex characters Ovaries implanted into males were transformed into testes Similar but less striking results were obtained in the decapod *Carcinus maenas* by Demeusy (1955, 1958) Neither gonad had any effect on host sexual characters and fragments of testes survived transplantation into females Ovarian fragments transplanted into young males regressed but were not transformed to testes The basis of these effects was established by Charniaux Cotton (1954b, 1955, 1956a,c,

1957, 1958b) She discovered in *O gammarella* a small gland on the vas deferens When this was implanted into females, it induced development of male secondary sex characters and conversion of ovaries to testes Complete removal of the gland from young animals prevents development of the characteristic male appendages, and if the appendages are removed from the mature male after gland extirpation they then regenerate in a juvenile form If an ovary is implanted into the neutral animal, the ovary develops yolk in the oocytes and with the next molt oostegites develop and form ovigerous fibers Charniaux Cotton concludes therefore that the gland, which she calls the androgenic gland is responsible for development of the testes and the male secondary characters, while the ovary is responsible for development of both permanent (oostegites) and temporary (ovigerous fibers) female secondary characters In both cases the effects are hormonally mediated The development of the ovary in genetic females is regarded as autonomous but development of a testis requires the vas deferens gland (Charniaux Cotton, 1959b) Carlisle (1959b) has suggested that the term "vas deferens gland" is preferable to androgenic gland" since it is based on anatomical relations rather than on presumed function Charniaux Cotton (1956b,c, 1958 a b) has found the vas deferens gland in the decapods *Clibanarius misanthropus* (Anomura) *Carcinus maenas* (Brachyura) and *Lysmata seticaudata* (Natantia) In *L seticaudata* the structure is present in the male phase but disappears completely before transformation to female Charniaux Cotton (1959a) considers that these animals are all genetic males which can function as females after involution of the vas deferens gland A similar change occurs in *Pandalus borealis* (Carlisle, 1959b c) The cause of this loss is not clear, but it is evidently responsible for the sexual conversion normal in these species

Veillet and Graf (1958) and Graf (1958) have studied the postembryonic development of the vas deferens gland in *Orchestia cavimana* Both sexes have identical gonads and anlagen of the vas deferens gland and genital duct at hatching In genetic males the vas deferens gland grows and this is followed by spermatogenesis and development of male secondary and accessory characters Balesdent and Veillet (1958b) report that hermaphroditic forms of this species can be converted to males by implants of the vas deferens glands Demeusy and Veillet (1958) have observed hypertrophy of the vas deferens gland of *Carcinus maenas* following eyestalk removal This is consistent with Demeusy's (1958) observation that eyestalk removal stimulates testicular maturation

In several terrestrial isopods Legrand (1954a,b,c, 1955a b, 1958b, 1959) showed that implantation of testes into females causes in contrast with the results in other groups, development of male secondary sex characters and regression of the ovary Implantation of ovaries into males as in other crustaceans is followed by regression of the implant and has no effect on

the secondary sex characters The interstitial cells of the testes exhibit cyclic transformations related to spermatogenesis In transplanted testes spermatocytes disappear, but the interstitial cells remain Consequently it appears that the latter cells are the source of the male hormone If partial testicular implants are made into females, these are effective only if the interstitial tissue is included The production of sex hormones by the ovary is shown by the fact that ovariectomy during sexual inactivity prevents oostegite formation whereas reimplantation of ovarian tissue induces development of these structures It seems likely that in amphipods and in decapods the vas deferens gland is homologous with the interstitial testicular tissue of the isopods The isopod *Asellus aquaticus* (Balesdent Marquet, 1958) *has a distinct androgenic gland, which, however, is not located in* the same anatomical position as in other crustaceans Recently Legrand (1958a) has noted that isolated virgin females of *Porcellio dilatatus* molt infrequently and exhibit no development of secondary female characters for as long as three years Copulation however induces rapid development of the oocytes and provokes a parturial molt This suggests a hormonally mediated response

c Conclusions At present homologies and analogies in the control of sexual functions in crustaceans and insects are almost entirely lacking In insects there appear to be both inhibitory and stimulatory factors acting through the corpora allata on vitellogenesis as such, though not on ovarian development In crustaceans there is an ovary inhibiting factor which acts on ovarian development, and the evidence for control of vitellogenesis is still very limited The thoracic glands of insects seem to inhibit the corpora allata and hence vitellogenesis, but the evidence concerning the ventral glands is contradictory The single observation that the ovary inhibiting substance of the queen bee, an ectohormone or pherormone has properties similar *to those of the ovary inhibiting hormone of the crustacean eyestalk* is of considerable interest There is no evidence for an internally active ovary inhibiting hormone in any insect

In the crustaceans there is clear evidence for gonadotropins originating in the eyestalks and for sex hormones *from the vas deferens gland and the* ovaries The eyestalk and ovarian factors stimulate development of female secondary characters The vas deferens factor is a typical male sex hormone It stimulates both spermatogenesis and development of male secondary characters It also inhibits ovarian development *No sex hormones have* been found in insects

3 The Control of Color Change

Two types of color change, characterized as morphological and physiological, are generally distinguished in animals Morphological color change

involves a change in the amount of pigment present in particular r
of the body surface Physiological color change involves a change in
of aggregation of pigment which is typically present in specialized ch
tophores and may be dispersed throughout these large highly branche
or concentrated in the center portion Insects rarely show either t
color change whereas both are reasonably common in crustaceans
of the work on control of color change has been concentrated on the c
of physiological color change in the crustaceans, but there has been
work with insects as well Color change in insects has been review
cently by Dupont Raabe (1957) and in crustaceans by Knowles (
Knowles and Carlisle (1956), Kleinholz (1957) and Carlisle and Ki
(1959)

a Insects *i Morphological color change* We have already discus
connection with molting and metamorphosis the morphological
change that precedes pupation in certain lepidopterans, and we sh
consider this problem further here Another type of morphological
change is that which occurs in *Locusta migratoria* (Orthoptera) This
which has been much studied recently in connection with efforts at de
ment of control measures, exists in two forms The solitary form is
and relatively sedentary in habits dense crowding induces a transfor
to a gregarious form, which is dark in color and exhibits charact
'marching' behavior The color change has an endocrine basis Nic
(1954) has injected blood from gregarious larvae into solitary larv
induced color change to the gregarious type The effective agent is s
in ether L Joly (1954) finds that implants of corpora allata into greg
type larvae change the color to that characteristic of the solitary for
that the effectiveness increases with size of the glands If the corpus a
is implanted just below the epidermis, out of contact with the bloo
color change occurs only in the region of the implant, so the action is
The essential condition for the development of green color seems to
presence of an active corpus allatum at the time of molt Implants of c
allata from *L migratoria* will cause transformation of *Acrida turrit*
a sandy to a green color Both colors occur in nature, depending on
ground (L Joly, 1955 P Joly, *et al* 1956 P Joly 1958) The sol
of the effective agent in ether and its origin from the corpora allata s
that it may be the juvenile hormone

ii Physiological color change The walking stick insect *Carausius* (
ippus) *morosus* (Orthoptera) exhibits a physiological color change
diurnal rhythm The epidermal cells contain melanin, yellow-orang
chromes, and a green pigment The melanin moves from the base
surface of the cell at night and in the other direction in the daytim
lipochrome is concentrated near the nucleus in the day dispersed th

tors concentrating black pigments and dispersing red pigments and are rather widely distributed through the central nervous system

The question of the number of chromatophorotropins has been much studied from the beginning of work in this field, and biological evidence indicating the existence of four to six specific factors was accumulated In recent years, the technique of electrophoresis on paper has been used by Carlisle, Knowles, and collaborators (Carlisle, 1955, Carlisle *et al*, 1955, DeLerma *et al* 1955, Knowles *et al*, 1955, Knowles, 1956, Knowles and Carlisle, 1956, Carlisle, 1958, Carlisle and Knowles, 1959), by Fingerman and collaborators (Fingerman, 1958a, 1959b, Fingerman and Aota, 1958c, d,e, Fingerman and Lowe, 1957b, 1958, Fingerman *et al*, 1958d, Fingerman, 1959c, Sandeen and Fingerman, 1959), and by Stephens *et al* (1956) This technique has proved quite successful as a means of separating specific factors The principal difficulty in attempting to compare the results of the various groups of investigators lies in the nature of the test animals Carlisle's group used the prawn *Leander serratus*, and Fingerman used the dwarf crayfish *Cambarellus shufeldti*, one cannot, without careful cross testing of known substances, assume that a substance which concentrates the red pigment in one species, for example, is the same as one which con centrates the red pigment of another species It would help considerably if those who are interested in this problem could standardize their testing procedures by cross tests of the same preparation on the respective test animals

Carlisle's group has separated three substances, and Fingerman's group four, as far as one can tell from the evidence available, it seems likely that two of Carlisle's substances are related to two of Fingerman's Carlisle's A substance is electropositive, and concentrates red pigment in the large chromatophores of *L serratus* It occurs in the postcommissural organs of Natantia and Stomatopoda, in sinus glands in these groups and in Reptantia, and in the corpora cardiaca of insects Fingerman has likewise found an electropositive red concentrating factor in the brains of two crayfish species and the prawn *Palaemonetes vulgaris*, it also occurs in the optic ganglia, but not in the sinus glands of the prawn, wherein it differs from Carlisle's A substance Östlund and Fange (1956) have made some progress in purifying a red concentrating factor by chemical means, this also may be related to the A substance Carlisle's B substance is electro negative, disperses red pigments in *L serratus*, and has been found only in the postcommissural organs of Reptantia and Natantia, Fingerman finds a similar substance in the brains of crayfish and prawns

Carlisle has also separated an A′-substance, which is electropositive and concentrates white pigment, from the postcommissural organ Fingerman has evidence for a white concentrating factor from the central nervous

is in a dark adapted state, but no such uptake in the light adapted state Injections of eyestalk extracts into dark adapted animals inhibited the up take This suggests that the hormones controlling light adaptation also have effects on permeability Koller (1955) also reports that extracts of decapod crustaceans kept in light accelerate gut movements whereas extracts from dark adapted animals slow gut movements

c Conclusion Parallels between control of color change in insects and crustaceans are few, primarily because the phenomenon itself is so rare in insects The isolation, at least to a stage of physiological purity, of chroma tophorotropic hormones has made substantial progress with the aid of paper electrophoresis, but comparison of results from different laboratories would be greatly facilitated by development of standardized test proce dures The presence of chromatophorotropins in insects which do not exhibit color change, taken with random observations indicating that factors active on chromatophores have other physiological actions as well, makes it seem probable that the basic action of the substances we call chromatophorotropins may be on some more fundamental process than dispersion or concentration of pigments in chromatophores Partial purifi cation of the agents in question should make possible a test of this hypothe sis

4 The Control of Metabolism and Other Functions

The concept of hormones as biocatalysts, based primarily on the striking effects of minute quantities of these substances, has long nourished the hope that the mechanism of endocrine action will be discovered in specific relation with enzyme systems This postulated relation gives special interest to hormonal effects on metabolic processes, but the study of such effects has thus far been quite disappointing, even in the vertebrates where hor monal control of various aspects of intermediary and total metabolism is well established One is led to consider whether hormonal actions in general are exerted upon more subtle aspects of cellular structure and activity, and metabolic control is a consequence of this type of action rather than of any influence on enzymes as such In the arthropods there has accumu lated a considerable body of evidence that interference with the neuro endocrine system has consequences in the metabolic processes of the animals There has been no satisfactory general evidence that basic meta bolic events are under neurohumoral control

a Insects i Pupal diapause and the thoracic gland The closest approach to demonstration of a specific metabolic action of an insect hormone is in the brilliant studies of Williams and associates on diapause in cecropia silkworm pupae The developmental aspects of these studies were discussed earlier The diapausing pupa has a very low rate of oxygen consumption, and the

onset of adult development is accompanied by a sharp increase in t value Schneiderman and Williams (1954a,b) and Schneiderman (19 1957) have shown that in the diapause condition the terminal oxidase tissue respiration is a phenol oxidase system whereas in active larv imagines, and pupae after termination of diapause, the terminal oxidas cytochrome oxidase Their initial conclusion was that the cytochro oxidase system disappeared from diapausing pupae and reappeared initiation of adult development Harvey and Williams (1958a,b) h examined the sensitivity of the heart of cecropia to cyanide and carl monoxide in diapause They conclude that, in the diapausing pupa cy chrome oxidase is still present but cytochrome c has virtually disappear Kurland and Schneiderman (1959) have also found that the missing co ponent in diapause is not cytochrome oxidase but cytochrome c Since evidence clearly proves that the initiation of adult development is cau by the hormone of the thoracic gland and since the reappearance of cy chrome c is the first sign of such initiation, the obvious implication is t the thoracic gland hormone acts directly to initiate cytochrome synthe There is however, no evidence to support the view that this is the b mechanism of the action of the thoracic gland hormone in other insects in its other effects

ii The corpus allatum and metabolism Considerable attention has b given to the role of the corpus allatum hormone in regulation of metaboli In discussing control of reproduction we pointed out that this hormon essential to vitellogenesis, and E Thomsen suggested years ago that effect may be more a metabolic effect than one specific to reproducti The effects of allatectomy are numerous and include increases in fat a carbohydrate content especially in the fat body, and increased oxy consumption L'Hélias (1955a) considers that the increased oxygen c sumption in *Dixippus* (= *Carausius*) *morosus* is the result of operat shock rather than of any basic changes in metabolism Novák (1959) f that implantation of corpora allata into castrated *Pyrrhocoris apterus* d not decrease oxygen consumption, but Samuels (1956) reports a disti increase in the oxygen consumption of the thoracic muscles of *Leucoph maderae* after allatectomy Schroder (1957) on the basis of studies of oxy consumption of tissue homogenates from *Leptinotarsa decemlineata*, c siders that the corpora allata elaborate a hormone stimulating oxy consumption at the end of diapause and one with inhibitory effects dur diapause His evidence does not seem adequate to establish this vi Lukoschus (1955a 1956b) points out the correlation of oxygen consumpt and size of corpora allata in the development of various castes of the hon bee *Apis mellifica*, but E Thomsen and Hamburger (1955) find no si correlation in the blowfly *Calliphora erythrocephala* They find no eff

on oxygen consumption of ovariectomy, confirming Thomsen's earlier view that the effect of the corpus allatum on oxygen consumption is direct

L'Hélias (1954a,b, 1955d, 1956a,b) has studied in some detail the metabolic events in *Carausius* (= *Dixippus*) *morosus* after removal of the corpora allata or corpora cardiaca Removal of the corpora cardiaca is followed by an increase in the reducing values of tissue extracts (which is equated with tissue carbohydrate) and in organic phosphate, and a decrease in tissue apyrase activity There is also a temporary increase in tissue ribonucleic acids and in uric acid content The latter is considered to result from increased production and decreased elimination of protein

In general the effects of the removal of the corpora allata are more striking There are increases in tissue carbohydrate involving a twofold increase in glycogen, tissue lipids, tissue amino acid, and tissue organic phosphate The ratio of protein nitrogen to total nitrogen decreases, as do tissue inorganic phosphate, alkaline phosphatase activity, and oxygen consumption Tissue uric acid decreases while the Malpighian tubules are "stuffed to bursting," presumably with uric acid The blood reducing value, which may be an index to the blood sugar, is decreased On the basis of these observations, L'Hélias concludes that the corpus cardiacum hormone increases catabolism of carbohydrate and protein while the corpus allatum hormone increases carbohydrate catabolism but stimulates protein synthesis She is inclined to the view that these effects on carbohydrates are mediated through the phosphatases, but her evidence is adequate to support these views only as a working hypothesis, and further dynamic studies of the effects of extirpation, implantation, and injection of extracts are required to substantiate the hypothesis

E Thomsen and Møller (1959) have reported briefly on the effects of removal of the medial protocerebral neurosecretory cells from *Calliphora erythrocephala* Vitellogenesis failed in such animals, and the intestinal proteinase activity decreased regardless of diet The authors conclude that failure of vitellogenesis may result from decreased protein synthesis

Nuñez (1956) has described experiments in *Anisotarsus cupripennis* larvae demonstrating a hormonal control of water balance Ligatures behind the head result in a marked uptake of water through the integument in the posterior portion of the body Section of both esophageal connectives or removal of brain and corpora cardiaca have the same effect, and extracts of the brain and corpora cardiaca but not of the subesophageal ganglia will stop the water uptake In the related insect *Blaptica* sp , which is unable to take up water through the epidermis, ligated larvae are less able to recover their weight following saline injections than are controls The same phenomenon was demonstrated in *A cupripennis*

iii Control of muscular activity Gersch and collaborators (Gersch *et al* ,

1957, Gersch, 1958a,b) have utilized effects on heart rate as test procedures for their neurohormones The crystalline products act in opposite senses on the hearts of *Periplaneta americana* Neurohormone C decreases amplitude, and in sufficient concentrations causes a systolic arrest, neurohormone D increases amplitude and causes diastolic arrest (Unger, 1956, 1957, Gersch, 1958a) Gersch has shown a parallel between the effects of nervous stimulation and those of neurohormone D in corethra larvae, suggesting that this hormone is the normal mediator of nervous stimuli to the heart These hormones also have actions on frog hearts and on color change in corethra and *Carausius morosus*, and are not identical with known vertebrate neurohumors The spider *Coelotes atropos* also has a neurohormone in the central nervous system which accelerates the spider heart and increases the amplitude of the frog heart (Gersch and Althaus, 1959)

Wigglesworth (1954c) has reported briefly on a similar neurohumor from insects with actions on cockroach hearts, movements of the Malpighian tubules, and the hindgut of locusts, and the chromataphores of prawns This product is found only in the corpora cardiaca and not in the brain or nerve cord, however Koller (1955) also reported that extracts of brain and corpora cardiaca of insects would accelerate the movements of Malpighian tubules and inhibit gut movements but that extracts of intestine and corpora allata stimulate gut movements As was noted earlier, the inhibitory effect was found in extracts of light adapted crustaceans, and the excitatory effect was evident in dark adapted forms

Harker (1954 1955, 1956) has studied the hormonal control of the diurnal activity rhythm of the cockroach *Periplaneta americana* The rhythm persists in constant darkness for at least 3 days, darkening of the eyes decreases this period If an animal exhibiting a regular rhythm is united parabiotically to one which has lost the rhythm both then show the diurnal rhythm The rhythm can also be evoked by transplanting the subesophageal ganglion The ocelli are the normal receptors for the optic stimuli which maintain the pattern of activity, and presumably the effect is mediated through neurosecretory elements of the subesophageal ganglion Ozbas and Hodgson (1958) find that extracts of cockroach (*Periplaneta americana, Blaberus cranufer*) corpora cardiaca will increase the activity of the isolated nerve cord and alter the behavior of the intact animals They consider that the active principle in their extracts originates in the neurosecretory cells of the brain

b Crustaceans The literature on hormonal control of metabolism in crustaceans has been reviewed by Knowles and Carlisle (1956), Scheer (1957), and Carlisle and Knowles (1959) The major effects established before the period of the present review were (1) a diabetogenetic effect of eyestalk extracts increasing blood sugar, (2) an effect on oxygen consump

tion, in that eyestalk removal increases total metabolism, (3) a possible factor in the eyestalk which shifts protein metabolism in the direction from catabolism toward anabolism, (4) an eyestalk factor which prevents calcium mobilization and possibly phosphate mobilization as well In the period covered by the present review, relatively little progress has been made in defining these actions more thoroughly Scheer and Scheer (1954b) were unable to demonstrate a marked increase in oxygen consumption following eyestalk removal from the Naples race of *L serratus*, which also shows no acceleration of molting after eyestalk removal Using *Carcinus maenas*, Altmann (1959) has found the expected increase in oxygen consumption following eyestalk removal, and in addition reports that injections of eye stalk extract depress oxygen consumption McWhinnie and Scheer (1958) have shown that the reducing value of crab blood is not a good index of blood glucose, which amounts to less than 20% of the blood sugar indi cated by the reducing value These authors were unable in a limited sample to demonstrate any effect of eyestalk removal on blood glucose, but Mc Whinnie and Saller (1960) have since shown that this operation was followed by a decrease in true blood glucose in the crayfish *Orconectes virilis* in summer Scheer (1959a,b, 1960) has shown that eyestalk removal from intermolt crabs [*Carcinides* (= *Carcinus*) *maenas*] brings about a premature increase in total body carbohydrate comparable to that which normally occurs in late intermolt and early premolt In prawns (*Leander xiphias*) and shrimps (*Processa acutirostris, P edulis*), which do not normally show any increase in carbohydrate content preceding molt, eyestalk removal causes increases to levels not normally observed in these animals, but characteristic of premolt crabs The question of possible identity of the factors regulating oxygen consumption and carbohydrate metabolism with the molt inhibiting hormone is not brought much nearer to an answer by these results Needham (1956) notes an increase in nitrogen excretion previous to the molt in the isopod *Asellus aquaticus* and discusses the question whether this may be controlled by the molt inhibiting hormone as well

Rudloff and Veillet (1954) can find no relation between the increased fat content of hermit crabs (*Pagurus diogenes pugilator*) parasitized by the rhizocephalan *Septosaccus cuenoti*, the feminization produced by this parasite and the fact that female hermit crabs normally have more fat than males Lenel (1957) has followed up his earlier work showing an effect of an eyestalk factor on deposition of carotenoid pigments in the epidermis of *Carcinus maenas* by showing that eyestalk removal increases deposition of the oxidized carotenoid astaxanthin specifically This is independent of dietary carotenoid (Lenel, 1959)

Carlisle (1955) has shown that the increase in volume of *Carcinus maenas*

at the time of molt is much greater than normal in eyestalkless animals This had been demonstrated before but Carlisle was able to show that sinus gland extracts taken from animals in any stage in the intermolt cycle will reverse the effect and that eyestalkless animals show a decreased resistance to hypotonic solutions in any stage of the intermolt cycle This leads him to postulate a water balance hormone which is not identical with the molt inhibiting hormone Lehman and Scheer (1956) showed that eyestalk removal causes a sharp increase in the uptake of radioactive phosphate from the medium by the crab *Hemigrapsus nudus* in the intermolt, but not in premolt The uptake occurs through the digestive tract and is probably a consequence of increased drinking of water In any case the endocrine factor involved is not the same as Carlisle's water balance principle, since it is not active throughout the intermolt cycle Nagano's (1958) evidence of a relation between dark adaptation and dye uptake through the gills of the crayfish was noted earlier

Koller and Kuhnen (1957) have separated from the crayfish *Astacus astacus* and the shrimp *Crangon crangon* two substances with opposite actions on the guinea pig ileum, one with a positive myotropic action, is found in the brain, the other, with negative action in the eyestalk Study of their relation to the neurohormones of Gersch (1958b) would be of interest Valente and Edwards (1955) have shown that eyestalk removal converts the diurnal activity rhythm of the crab *Tricodactylus petropolitanus* to a pattern of slow arrhythmic activity, and injection of eyestalk extracts stops all activity Rasmussen (1959) notes that parasitization of male *Carcinus maenas* induces a migration into deep water when the parasite matures which is comparable to the migration of breeding female crabs He supposes that the effect is hormonally mediated

c Conclusions The studies of metabolic control in arthropods still do not have the definition and neatness seen in other branches of arthropod endocrinology or in the work on metabolic control in the vertebrates In part this is the result of a lack of purified preparations of the hormones involved in part the lack of adequate knowledge of the metabolism of the animals concerned and in part the small amount of effort expended on the problem to date The single clear cut metabolic action, that of the thoracic gland hormone in eliciting cytochrome synthesis in diapause termination in cecropia, appears to be a highly specialized case Of the other effects we need only note the rather remarkable similarity between the effect of removal of the corpora allata from insects and of removal of eyestalks from crustaceans In both cases the oxygen consumption increases, tissue carbohydrates increase, blood sugar decreases, and protein catabolism increases relative to anabolism One is tempted to wonder about a relationship between the juvenile hormone, the molt inhibiting hormone and these

various actions, particularly since Schneiderman and Gilbert (1958) report that the crustacean eyestalk is the richest source of juvenile hormone outside the insect group There also appears to be in both insects and crustaceans a factor that restrains water uptake, which may or may not be related to the above

Consideration of neurohormones with actions on heart and smooth muscle brings us into a realm which is in part outside our defined scope and which has been reviewed by Welsh (1957) elsewhere These hormones have been mentioned here because the limited evidence now available shows that two such substances in pure form have actions not only on heart and muscle but also on color change

IV Assay and Purification of Arthropod Hormones

Various aspects of this problem have been reviewed by Butenandt (1955, 1959), Carlisle and Knowles (1959), Gersch (1957, 1958a), Karlson (1956a,b), Knowles and Carlisle (1956), and Novak (1959) It is clear that within the period covered by the present review we have passed from a phase of research in which the emphasis was on qualitative studies of the consequences of ligation of body regions, extirpation of whole organs, and implantation of organs or injection of crude extracts, into a phase in which increasing emphasis is placed on isolation of purified hormones and quantitative studies of their actions Much remains to be learned from the older types of experiment, but progress will be accelerated to the extent that deductions from such experiments can be tested with pure chemical entities

The chemical problem of the isolation of arthropod hormones is probably a more difficult one than that faced by the pioneers in the isolation of vertebrate hormones a generation or more ago because most of the arthropod hormones are probably neurosecretory in origin These are formed in a more or less continuous secretory process rather than by accumulation in a single endocrine organ readily available in large quantities from commercial sources On the other hand we have at our disposal the experience of a generation of chemists and an armamentarium of remarkably effective methods of purification

The first requisite of any kind of purification attempt is an adequate assay method The assay should be simple to perform, reproducible, and quantitative The classic form of quantitative relationship, and the simplest to work with, is that in which the response is directly proportional to the logarithm of the dose, but most of the assays used so far are threshold assays, in which the minimum dilution that will bring about a specific effect is determined The assay should be as sensitive as possible and should depend upon a normal response to the substance under study With assays of this type it should be possible even for modestly equipped laboratories to carry

out isolations of small amounts of material to the stage of physiological purity, in which a tissue or organ extract having several physiological actions is separated into components, each of which has a set of constant reproducible actions Purification to the stage of chemical purity will in general require the facilities of laboratories especially equipped for isolation of minute quantities of substances from large volumes of starting material, and experienced in such isolation It is to be hoped that more laboratories so equipped will take an interest in the arthropod material The cost of such studies is great, but one might predict on the basis of experience with plant hormones that isolation, purification and determination of the structure of arthropod hormones would lead to important commercial applications, particularly in control measures

1 *Ecdysone*

The isolation by Butenandt and Karlson (1954) of a pure crystalline substance capable of inducing metamorphosis in insects constitutes the first isolation of a hormone from any invertebrate (Williams, 1954, Butenandt 1955 1959 Karlson 1956a,b) The biological activity of this material was discussed earlier Its chemical nature has not been determined as yet beyond the crude formula ($C_{18}H_{30}O_4$), certain physiological properties, and some indications as to the presence of an alpha beta unsaturated ketone and a carbonyl radical It does not appear to be a steroid contains no nitrogen and does not resemble any known hormone The assay method used in the isolation utilizes the initiation of pupation in the larvae of *Calliphora erythrocephala* Larvae are ligated behind the head and after 24 hours those individuals in which only the head has pupated are selected The head is cut off these and the solutions to be tested are injected into the abdomens The abdomens are then examined for the color change which indicates initiation of pupation A *Calliphora* unit is defined as the amount of material that will initiate pupation in 50–70% of larval abdomens One unit of α ecdysone is equivalent to 0 0075 μg A second material, β ecdysone, is also found in extracts and has an activity of one unit to 0 015 μg A related material has been purified from the prawn *Crangon vulgaris*

Kobayashi (1958) and Kobayashi and Kirimura (1958) report progress in purification of the brain hormone responsible for activating the thoracic gland The details are not yet available

2 *Neurohormones of Gersch*

Gersch and collaborators (Gersch and Mothes 1956, Gersch, 1957, Gersch and Unger, 1957, Gersch *et al*, 1957, Gersch, 1958a) have reported the isolation in crystalline form of two substances from the central nervous system, including the corpora cardiaca of cockroaches (*Periplaneta ameri*

cana) They give no information as to the chemical nature of these substances beyond the lack of identity with epinephrine, acetylcholine, or histamine Their assay procedures include effects on the hearts of cockroaches, the melanophores of *Dixippus* (=*Carausius*) *morosus* and of the corethra larvae, and of the frog heart They give no quantitative data on the technique of assay in the publications available to me As starting material for isolation of neurohormone D, they used the dissected nervous systems of 320 cockroaches, and they report a yield of 2 μg, which is remarkably high by comparison with Karlson's yield of 25 μg of ecdyson from 500 kg of silkworm pupae In response to a query from Buckmann on this point Gersch (1958a) attributed the high yield to use of the active tissue rather than the whole animal as starting material and to a possibly higher hormone content On the basis of their physiological actions it seems likely that the neurohormones of Gersch are the substances responsible for the color change in *C morosus* and corethra, and that they may have a normal role in the regulation of heart rate as well It will be of interest to see a comparison with the C substance of Carlisle *et al* (1955) and Knowles *et al* (1955) and the fluorescent substances observed in extracts of *Hydrous piceus* corpora cardiaca by De Lerma *et al* (1955) or with the myotropic substances from the brain and eyestalk of crustaceans (Koller and Kuhnen, 1957)

3 The Juvenile Hormone

Williams (1956b 1957) has reported some progress in purification of the juvenile hormone of insect corpora allata, and Williams *et al* (1959) have found that extracts of many mammalian tissues have juvenilizing action Williams' assay and that of Gilbert and Schneiderman (1957) depends on injections of serial dilutions of extracts into lepidopteran pupae, usually cecropia, and an evaluation of pupal characters in those individuals which molt soon after the injection Schneiderman and Gilbert (1957) report partial purification of the hormone and its occurrence in large amounts in the abdomens of senile males of several lepidopteran species Williams (1956b) also noted this latter phenomonen Gilbert and Schneiderman (1957, 1958c) and Schneiderman and Gilbert (1958) have improved the assay method by implanting a solution of the hormone in wax into the integument of the pupa Active substances then produce an island of pupal tissue at the site of the implant These authors provide evidence that pupae and adult females of cecropia are able to inactivate the hormone when it is injected Juvenile hormone action has been found in extracts of several invertebrates The richest source outside the insects is the crustacean eyestalk Wigglesworth (1958) has developed an assay using stage V larvae of *Rhodnius prolixus* The cuticle is abraded and graded dilutions of extracts

are applied to various regions, the appearance of these regions is then examined following the imaginal molt He has also used pupae of *Tenebrio molitor*, introducing minute droplets of extracts through a puncture in the cuticle The juvenile hormone is ether soluble and resistant to boiling alkali

4 Crustacean Chromatophorotropins

In the earlier discussion when we noted that Carlisle Knowles and associates (Carlisle *et al*, 1955 De Lerma *et al*, 1955, Knowles *et al*, 1955, Knowles 1955, Dupont Raabe, 1957) have had considerable success in separating by electrophoresis on paper the chromatophorotropins of crustaceans and insects to a state of physiological purity at least Fingerman and collaborators (Fingerman and Lowe, 1957b Fingerman, 1958a, 1959b, Fingerman and Aota, 1958c e, Fingerman *et al*, 1958d) and Stephens *et al* (1956) have had success with the same method It does not however offer promise of isolation in sufficient quantities for chemical study, though other forms of preparative electrophoresis might be applied Östlund and Fange (1956) reported some progress of purification of a chromatophorotropin by chemical methods, but they have not yet reported a pure product The assays used by all the workers in this field depend on visual grading of pigment dispersion in the chromatophores While there is an element of subjectivity in such grading, it is remarkably accurate and reproducible Fingerman and Aota (1958e) have obtained linear log dose response curves with this method Knowles (1956) has discussed the criteria for assay of these factors Purification is complicated by the fact that certain tissues are able to inactivate the hormones (Fingerman and Lowe, 1957b) There is very little evidence concerning the chemical nature of these hormones De Lerma *et al* (1955) found fluorescence in the active bands of chromatograms of the sinus gland in corpora cardiaca extracts but the spectra did not coincide with those of known substances L'Hélias (1955b,c, 1956a b) has argued on the basis of the presence of fluorescent substances related to folic acid in glandular extracts and evidence that certain pterins (xanthopterin) induce color change in *Carausius morosus*, that the active hormones are pterin derivatives The evidence is not adequate to support this conclusion at present

Fingerman *et al* (1958a b 1959a b) have utilized electrophoresis to separate light adapting and dark adapting hormones from extracts of crustacean nervous organs The hormones are assayed by observing effects on movements of the distal retinal pigment in the eye, the measurements can readily be made quantitative It would be of interest to know whether these hormones are identical with any other chromatophorotropins

V General Summary and Conclusions

In this concluding section, I shall attempt to summarize the more important developments of the last five years in terms of the major subdivisions of the neuroendocrine system, stressing points of resemblance and difference among the various arthropods

1 The Neurosecretory System

The morphological pattern of the neurosecretory system of arthropods shows a general similarity throughout the group, with concentrations of cells in the protocerebrum, tritocerebrum, and the ganglia of ventral nerve chain In each region there are possibly several morphologically distinct groups of cells It will be an important task for comparative histologists to define and compare the cell types of different arthropods and different species in the various arthropod classes, to serve as a basis for physiological study

The physiology of the neurosecretory cells has thus far been very poorly explored Two techniques—correlation of histological changes with physiological events, and selective extirpation—are available It is most productive to use these in combination, such studies have been few The present evidence suggests that the medial protocerebral cells of insects provide the basic stimulus to initiation of molt, termination of diapause, and possibly to initiation of vitellogenesis There is some evidence for a similar effect on molt initiation from the crustacean eyestalk, but this requires further study The medulla terminalis cells of crustaceans form a molt inhibiting hormone, there is no evidence as yet for such a hormone in insects Certain protocerebral cells appear to be responsible for inhibition of egg production in starvation or other conditions in certain insects The medulla terminalis cells of crustaceans form an ovary inhibiting hormone, this however, appears to act directly on the ovarian tissue rather than on vitellogensis Chromatophorotropins are formed throughout the central nervous system of crustaceans, only the tritocerebral region of the insect brain has been identified as the source of such substances, but two substances having chromatophorotropic activity have been crystallized from an insect central nervous system Neurosecretory cells in the subesophageal ganglion in insects appear to be able to activate the corpus allatum in the response to feeding in some insects, or ovariectomy in others These may be related to or distinct from the protocerebral cells, but cyclic changes related to reproductive activity are seen in similar cells throughout the crustacean central nervous system In this collection of rather miscellaneous information there is clearly room for extensive comparative study

2 The Neurosecretory End Organs

The axons of the neurosecretory cells of all arthropods examined thus far terminate in part in specialized end organs These may or may not have secretory cells in addition to the axon termini, and the issue is still disputed in some cases There is little good physiological evidence of intrinsic secretion in the corpora cardiaca of insects or in the sensory papilla (X) organ or the sinus gland of crustaceans, but the corpora allata of insects have a well established secretory function In the larval molts of insects the secretion of the corpora allata prevents development of imaginal characters, the effective agent has been partly purified and is known as the juvenile hormone In adult females the secretion of the corpora allata is essential to the deposition of yolk in mature oocytes, the effective agent has not been established, but may be different from the juvenile hormone The metabolic changes following removal of the corpora allata from insects strongly resemble those following removal of the eyestalks from crustaceans, and the crustacean eyestalk contains juvenile hormone There is evidence that the corpora allata, and the juvenile hormone, can activate the thoracic gland

3 The Endocrine Glands

The major endocrine gland of arthropods has been termed in this review the thoracic gland in insects or the ventral gland in crustaceans It has, as well, been variously known as prothoracic gland pericardial gland, peritracheal gland and the Y organ In nearly all insects and in one crustacean studied molt does not occur in its absence The onset of premolt in all the forms studied is associated with evidences of secretory activity in the thoracic or ventral gland, and extracts or implants of the gland will promote molt The thoracic gland is also involved in termination of diapause In one instance its effect is primarily to stimulate synthesis of cytochrome c The substance called ecdysone, isolated from insects in crystalline form, duplicates the action of the thoracic gland and a similar substance has been found in crustaceans It seems very likely, but is not proved, that this substance is in fact the hormone of the thoracic or ventral glands

In addition crustaceans have endocrine tissue which forms a male sex hormone In most forms this tissue occurs in a discrete gland on the surface of the vas deferens but in some it is incorporated in the testis The ovary of crustaceans also secretes factors responsible for secondary sex characters No evidence for the existence of sex hormones in insects has been published

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Effects of Hormonal Imbalances on Dietary Requirements

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I Introduction

Although a considerable volume of literature has appeared on the effects of nutritional deficiencies on endocrine function, much less work has been reported on the effects of hormonal imbalances on dietary requirements This chapter will deal principally with the effects of alterations in growth hormone thyroid, adrenal cortical and gonadal steroids on dietary needs Other hormones such as insulin and parathormone also influence nutrition but these will not be considered here

It is well known that the endocrine glands are important in regulating the metabolism of foodstuffs in the body Thus, growth hormone increases protein retention and decreases fat deposition, the thyroid influences the metabolism of all foodstuffs, the adrenal cortex helps regulate protein,

carbohydrate, fat, salt, and water metabolism, and gonadal hormones may influence the metabolism of proteins, vitamins, or other factors

Dietary needs may remain unchanged when the endocrine glands are secreting hormones within "normal" ranges However, when there is a marked excess or deficiency of some hormones in the body, the need for dietary factors may be altered This problem may have clinical as well as theoretical importance because of (1) the use of hormones in human and animal practice at times in large doses and over extended periods of time, and (2) the occurrence of spontaneous dysfunction of the endocrine glands If marked changes in endocrine balance can induce or aggravate dietary deficiencies, optimal dietary supplementation should help to alleviate the resulting clinical picture

II Growth Hormone

1 *Administration of Growth Hormone*

Administration of high doses of a potent growth stimulating agent such as anterior pituitary growth hormone would be expected to increase all dietary requirements that depend on the rate of growth, or more specifically, on the rate of protein synthesis No studies have been made to determine the actual change in dietary requirements, but accentuation of certain dietary deficiencies by high doses of growth hormone has been reported More commonly, lower, physiological doses of growth hormone have been used to determine whether growth can be stimulated in animals subjected to various dietary deficiencies Considerable evidence has accumulated indicating that growth hormone does not increase body weight unless nutritionally adequate diets are used The effects of exogenous growth hormone will, of course, vary with the dosages used, duration of injection period, and degree of severity of the dietary deficiency concerned

a Vitamin A One of the earliest studies in this field was that of Ershoff and Deuel (1945), who observed that injections of growth hormone preparations accentuated the signs of vitamin A deficiency, markedly increased the mortality rate, and decreased the average length of survival on the A deficient diet The vitamin A deficient rats failed to gain weight in agreement with previous findings by Margitay-Becht and Wallner (1937) Ershoff and Deuel (1945) suggested that the hormonal stimulus may have depleted body stores of the vitamin faster than would otherwise have occurred

b Choline Injections of anterior pituitary growth hormone increased the renal damage resulting from choline deficiency and did not prevent weight loss, however, there was no apparent increase in liver lipid accumulation (Hall and Bieri, 1953) Both the incidence and severity of de

generative renal changes were increased, and renal weight was further augmented This accentuation of choline deficiency was confirmed by Wilgram *et al* (1956), who found that under suitable conditions growth hormone precipitated first hemorrhagic lesions of the kidney and then cardiovascular lesions Growth hormone did not increase food intake of the choline deficient rats These authors considered it probable that the choline requirement was elevated because of the increased methionine requirement resulting from growth hormone stimulation of protein synthesis

c Pantothenic Acid Injections of growth hormone in adult female rats accidentally given a diet deficient in pantothenic acid precipitated an acute deficiency of this vitamin, indicated by loss in body weight and porphyrin deposition (Lotspeich, 1950) Pantothenic acid supplementation of the growth hormone injected animals resulted in a striking increase in body weight and disappearance of deficiency signs The low requirement of adult rats for this vitamin (Unna and Richards, 1942) was apparently increased by growth hormone injections Barboriak and Krehl (1957) recently reported that growth hormone injections in hypophysectomized, pantothenic acid deficient rats accentuated the characteristic adrenal cortical pathology found in corresponding uninjected deficient animals

Beare *et al* (1954) reported a decreased weight gain, but no reduction of nitrogen retention in pantothenic acid deficient rats given growth hormone However, Hazelwood *et al* (1955) observed a significant reduction of the nitrogen retaining effect of growth hormone in rats subjected to a mild deficiency of pantothenic acid The use of pair fed controls eliminated decreased food intake as a factor in the reduction of nitrogen retention Moreover, this decreased response to growth hormone injections persisted when the deficient animals were deprived of both adrenals, indicating that adrenal cortical steroids were not concerned

d Vitamin B_6 Growth hormone injections markedly accentuated vitamin B_6 deficiency (Beaton *et al*, 1952, Beare *et al*, 1953) Increased severity of the characteristic acrodynia and further increases in fasting blood urea levels were noted Failure to gain weight or loss in body weight also occurred, together with reduction of nitrogen retention The observed decrease of vitamin B_6 in the liver of growth hormone injected animals suggested a more rapid depletion or utilization of the vitamin in both B_6 deficient and supplemented rats

e Other B Vitamins In young hypophysectomized rats, increased body weight and bone growth induced by growth hormone were augmented by folic acid (Penhos and Foglia, 1954, Penhos and Fajer 1955) Growth hormone injections did not promote growth in riboflavin deficient rats (Colonge and Raffy, 1952, Beare *et al*, 1954), although Beare and co

carbohydrate, fat, salt, and water metabolism, and gonadal hormones may influence the metabolism of *proteins, vitamins, or other factors*

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workers (1954) noted a slight increase of doubtful significance in nitrogen retention Simpson and Evans[1] observed failure of growth hormone preparations to stimulate growth in B_1 deficient rats In disagreement with this finding is the report of Beare *et al* (1954) that B_1 deficient rats gained weight and retained nitrogen when injected with growth hormone These contradictory results can probably be explained by differences in the severity of deficiency Penhos and Foglia (1954) have reported that the increases in body weight and thymus weight induced in young rats by injection of growth hormone were further increased by vitamin B_1 supplements On the other hand, Meites *et al* (1957) found that neither vitamin B_{12} deficiency nor B_1 supplements influenced the action of growth hormone in promoting growth in either hypophysectomized or intact rats They concluded that under their experimental conditions growth hormone did not appear to increase the requirement for this vitamin Ershoff (1951a) reported that immature rats retarded in growth by thyroid feeding (see Section III, 1, c) did not respond to growth hormone preparations

f Lipids (Essential Amino Acids) In the absence of the essential unsaturated fatty acids (EFA), growth hormone injections did not promote growth (Deuel *et al*, 1950) However, dietary supplementation with 20 mg linoleic acid daily was followed by a response to administered growth hormone Deuel *et al* (1946) had previously reported that the administration of growth hormone to rats maintained on diets containing either butter fat or various vegetable fats and oils resulted in equivalent gains in body weight, i e in the presence of sufficient EFA the dietary fats used were of similar nutritional value

g Protein Numerous studies have demonstrated that an optimal response to growth hormone is dependent on both the quantity and quality of dietary protein Adult rats maintained on a 6% casein diet did not gain weight with growth hormone injections although some retention of nitrogen occurred (Gordan *et al*, 1947) Addition of DL methionine (66 mg daily) resulted in a markedly increased nitrogen retention and in a significant gain in body weight, the food intake was kept constant in these experiments The authors concluded that this action of methionine appeared to be related to the amino acid requirement for building new tissue rather than repair of hepatic damage, as no liver changes were noted histologically Further studies by Gordan *et al* (1948) showed that both nitrogen retention and weight gain varied concordantly with dietary protein content, 24% dietary casein being optimal for growth with the dose of growth hormone employed

Alcock (1934) reported that tryptophan deficient rats given growth

[1] Personal communication from H M Evans cited by Ershoff and Deuel (1945)

hormone gained weight whereas uninjected controls barely maintained their body weight With a more severe deficiency and a more highly purified hormone preparation, Bavetta *et al* (1956) found that administration of growth hormone for 10 days failed to increase body weight in rats fed a tryptophan low diet Growth hormone injections resulted however, in resumption of chondrogenesis and osteogenesis in the epiphyseal region of the tibia, bone formation in alveolar bone, and formation of normal appearing incisal dentine These effects were not due to increased food intake as growth hormone injected animals were pair fed with saline treated animals Similarly, Scott and Dynes (1957) reported that injection of growth hormone did not affect body weight of rats deficient in phenylalanine and tyrosine, but stimulated chondrogenesis, although not to the same extent as in pair fed controls

Chow and Greep (1948) reported that growth and survival of hypophysectomized rats given growth hormone varied with the biological quality of proteins fed during a 3 week period A basal purified diet containing 30% of a test protein was used Growth, food intake, and survival of operated rats on diets containing soya protein were significantly less than on diets containing lactalbumin, "vitamin free" casein, or "crude" casein Inasmuch as "crude" casein diets appeared to give the most favorable results, the possibility exists that trace nutritional factors in the test proteins may also have influenced the results These investigators also observed failure of growth hormone injected rats to survive longer than 2 weeks after hypophysectomy when given a protein free diet, but data on comparable uninjected animals are lacking

2 *Hypophysectomy*

Shaw and Greep commented in 1949 that "no systematic investigation on the nutritional requirements of hypophysectomized rats has yet been made " Unfortunately this statement is still correct

Inasmuch as excess growth hormone apparently increases the requirement for many dietary factors, the absence of growth hormone resulting from hypophysectomy would be expected to decrease these requirements Absence of other hormones such as thyroxine and adrenal cortical steroids might be expected further to decrease some of these requirements (however, see Sections III and IV) The marked decrease in food intake of hypophysectomized animals and a possible reduction in absorption would strongly suggest that a diet "richer" than that fed ad libitum to normal animals may be beneficial or necessary and that a diet "poorer" than usual might have more deleterious effects in hypophysectomized animals than in intact rats

Shaw and Greep (1949) reported that only 10% of immature Sprague Dawley rats maintained on a laboratory chow diet survived hypophysectomy beyond 45 days and that all succumbed after 63 days When purified diets were used, 60–70% of the animals lived for 88 and 112 days after the operation Body weight was also increased and some tail growth was observed

Many publications from the Institute of Experimental Biology in Berkeley have demonstrated that 90% of hypophysectomized Long Evans rats maintained on a stock diet of natural foodstuffs (wet mash of Diet I plus lettuce) survived for many months and that some lived for 1 5–2 years after the operation (Becks *et al*, 1946) B vitamin supplementation of this stock diet did not improve growth or survival of such animals nor did maintenance on a purified diet result in any significant improvement (Simpson and Nelson, unpublished)

The dependence of hypophysectomized rats on adequate food and caloric intake is well known In the absence of the pituitary, the rat cannot withstand fasting for an extended period because of rapid depletion of liver glycogen stores and the early occurrence of hypoglycemia with convulsions and death When hypophysectomized rats are force fed by stomach tube, increase in body weight can be observed (Samuels *et al*, 1943), but such gains are characterized by an accumulation of fa in the body The presence of ample tissue stores of essential nutrients in rats hypophysectomized at maturity may compensate to some extent for inadequate diets

3 Summary

Administration of high doses of growth hormone apparently increased the requirement and accentuated deficiencies of vitamin A, choline, pantothenic acid, and vitamin B_6 Presumably the effects of deprivation of total protein or of any essential amino acid would also be accentuated, as the available data show failure of growth hormone to stimulate growth on such diets Inability of growth hormone injections to increase body weight and promote nitrogen retention has also been shown to a variable extent in rats deficient in pteroylglutamic acid, riboflavin, thiamine, the "antithyrotoxic factor," and essential fatty acids

Little is known concerning the nutritional requirements of hypophysectomized rats However, the available data strongly suggest that commercially prepared diets may need to be supplemented to maintain hypophysectomized animals in good condition

III THYROID

1 Hyperthyroidism

a General Increase in Dietary Requirements Hyperthyroidism is generally considered to increase the need for all dietary essentials, including

protein, fat, carbohydrates, vitamins, and minerals This increased need is usually related to the increase in basal metabolism, appetite, food intake, and intestinal absorption induced by hyperthyroidism When body growth of young animals is used as an index of nutritional adequacy, an increased need for the following vitamins during administration of large doses of thyroactive substances had been demonstrated before 1943 (reviewed by Drill) vitamin A, thiamine, riboflavin, vitamin B_6, pantothenic acid, and ascorbic acid An increased requirement of vitamin D to prevent the negative calcium balance resulting from the increased fecal calcium loss during hyperthyroidism had also been shown (Pugsley and Anderson, 1934a, b)

Since 1943 an increased need for other essential dietary factors during hyperthyroidism has been demonstrated as follows pteroylglutamic acid in rats (Martin, 1947, Kelley *et al*, 1950) and chicks (Haque *et al*, 1948), choline and cystine in rats (Handler and Follis, 1948) vitamin E in chicks (Wheeler and Perkinson 1949) and in rats (Tentori *et al*, 1953), vitamin B_1 in rats (Betheil and Lardy, 1949, Emerson, 1949), mice (Bosshardt *et al*, 1949), and chicks (Nichol *et al*, 1949), unsaturated fatty acids (Greenberg and Deuel, 1950, Greenberg, 1952) magnesium (Vitale *et al*, 1957) and unidentified factor(s) in rats and mice (Ershoff, 1947, 1949a) The majority of these studies have been carried out with rats, but increased requirements during hyperthyroidism have also been demonstrated in mice, rabbits, guinea pigs, dogs swine, and chicks It is apparent, however, that many dietary essentials have not been studied during hyperthyroidism and that our knowledge of requirements for this condition is still incomplete Moreover, few quantitative studies have been made to determine the magnitude of the increased dietary requirement or to correlate this with the degree of hyperthyroidism For example, Pfander (1952) found that hyperthyroidism in the guinea pig which was sufficient to increase heart rate by 25% raised the ascorbic acid requirement by 45% Ziffer *et al* (1957) have recently reported that after a test load of 50 μg vitamin B_1, *hyperthyroid patients showed only* one *half the* increase in *blood* B_{12} and in urinary B_{12} excretion observed in euthyroid controls Hypothyroid patients with myxedema exhibited increased blood B_{12} levels and more than double the control urinary vitamin excretion following the test dose

On adequate diets a mild degree of hyperthyroidism may not significantly alter body growth and in some species, i e the mouse, may actually increase the rate of growth (Koger and Turner, 1943, Meites, 1953a) A more severe degree of hyperthyroidism however, inhibits body growth in the mouse and many other species and may even induce death, unless there is an increased intake of essential nutrients The protective effects of extra nutrients may be complete or only partial, depending on the degree of hyperthyroidism, duration of treatment, composition of the diet, age,

sex, and species Also, not all effects of thyroactive substances are counter acted by increasing the intake of nutrients

b Vitamin B_1 The extensive studies on vitamin B_{12} and hyperthy roidism serve to illustrate the findings with other vitamins The increased requirement for this vitamin during hyperthyroidism was recognized even before its isolation, and diets containing thyroactive substances had been used to bioassay vitamin B_{12} activity With standardized conditions, the growth stimulation of hyperthyroid rats, mice, and chicks is proportional to the vitamin B_1 dosage (Bosshardt *et al*, 1949, Robblee *et al*, 1948 Emerson, 1949) The maintenance of thymus weight in hyperthyroid rats was likewise proportional to the vitamin B_{12} dosage (Pentz *et al*, 1950) Vitamin B_1 supplementation of hyperthyroid animals has usually resulted in increased food intake and greater efficiency in converting food into body weight gains, e g Meites and Shay (1951) When food intake was held constant, vitamin B_{12} supplementation did not prevent losses in body weight of hyperthyroid rats, although urinary nitrogen was decreased (Rupp *et al*, 1951)

Not all effects of thyroactive substances are counteracted by increasing the intake of nutrients For example, neither vitamin B_{12} nor vitamin sparing antibiotics counteracted the increased basal metabolic rate result ing from high doses of thyroid, although growth inhibition was partially or completely prevented (Meites and Ogle, 1951, Meites and Shay, 1951) Vitamin B_{12} did not prevent adrenal hypertrophy (Pentz *et al*, 1950) nor depression of testis weight (Meites and Shay, 1951) resulting from hyper thyroidism Thyroid weight and I^{131} uptake in normal and hyperthyroid rats or in normal chicks were also unaffected by vitamin B_{12} (Meites, 1950b) or antibiotics (Libby and Meites, 1954) It is of interest that when mild hyperthyroidism was induced in mice (Meites, 1953a) or swine (Bee son, 1951) on a vitamin B_{12} deficient diet, body growth was greater than when the diet was supplemented with vitamin B_1 The reason for this apparent exception is not known

c Unidentified Factors ("Antithyrotoxic Factor") and Fat The need of the hyperthyroid rat for additional factors distinct from vitamin B_1 was first shown by Ershoff (1947, 1949b) Desiccated whole liver and extracted liver residue (the water insoluble fraction) completely counteracted the retardation in growth and decreased rate of survival resulting from incor poration of a high level of thyroid (0 5%) in a purified diet, whereas anti thyrotoxic factor (ATF) concentrates and crystalline vitamin B_1 were ineffective under these conditions (Ershoff, 1948 and 1949a) Whole liver powder was likewise beneficial for the growth and survival of hyperthyroid mice (Ershoff, 1949a) Many investigators have confirmed these findings and have found, moreover, that the beneficial effects of both vitamin B_1

and the antithyrotoxic factor in hyperthyroid rats varied with the composition of the diet and the degree of hyperthyroidism, e g Bolene *et al* (1950), O'Dell *et al* (1955), Overby *et al* (1959a)

The antithyrotoxic activity of other substances such as soybean meal and cottonseed oil has emphasized the importance of dietary fat in hyperthyroidism (Ershoff, 1949c and 1953b, Overby *et al*, 1959b) The protective effect of unsaturated vegetable fats varied with the linoleic acid content and reached a maximum with dietary levels of 15% cottonseed oil (Overby *et al*, 1959b) Greenberg and Deuel (1950) and Greenberg (1952) have shown that a mild degree of hyperthyroidism (0 05% thyroid in diet) accentuated a deficiency of unsaturated fatty acids With the more severe hyperthyroidism resulting from dietary levels of 0 5% thyroid, the linoleic acid requirement must be increased correspondingly It is possible that under such conditions additional calories supplied by fat may be necessary to counteract the loss of energy due to oxidation not coupled with phosphorylation (Overby *et al*, 1959b)

The growth stimulation of hyperthyroid rats by defatted liver residue and by linoleic acid containing fats appeared to be additive (Ershoff, 1953b, Overby *et al*, 1959b) so that the identity of the antithyrotoxic factor in liver residue has not yet been established The amino acid content of liver residue has been found to be inactive thus eliminating the protein portion of the residue (Overby *et al*, 1959c) There is also evidence that the antithyrotoxic factor in liver residue (and in plant residue Ershoff *et al*, 1959) may be identical with unknown factor(s) needed by rats given massive doses of estrogens, cortisone glucoascorbic acid, atabrine, promin, and other stress agents (e g Ershoff, 1955, Ershoff *et al*, 1959)

2 Hypothyroidism

a General Decrease in Dietary Requirements Hypothyroidism results in decreased basal metabolism, appetite, food intake, and intestinal absorption, and dietary requirements are usually considered to be decreased This is probably true in most instances, although quantitative evidence for this assumption is meager For example, Wiese *et al* (1948) reported that hypothyroidism produced by thiouracil feeding prolonged the survival of vitamin A deficient rats Liver stores of vitamin A were depleted more slowly when rats were treated with thiouracil (Johnson and Baumann, 1948, Arnrich and Morgan, 1954), suggesting a decreased need or utilization of the vitamin Growth retardation through caloric restriction also resulted in slower depletion of vitamin A reserves (Arnrich and Morgan 1954) Cooper *et al* (1950) have reported that thiouracil treatment decreased the vitamin A requirement of chicks It may be mentioned here that the most recent studies on the relation of thyroid status to the con

version of carotene to vitamin A have not provided any evidence that thyroid function affects this conversion in either rats or dogs (Arnrich and Morgan, 1954, Arnrich, 1955, Worker, 1956).

Hypothyroidism resulting from thyroidectomy, thiouracil, or sulfonamides prevented or delayed cirrhosis of the liver in rats and improved growth and survival of such animals (György and Goldblatt, 1945, Handler, 1948). Gyorgy and Goldblatt (1945) believed that the beneficial effects of thiouracil could not be explained by differences in food intake, whereas Sellers and You (1951) observed that restriction of food intake to the same degree as in thiouracil treatment also delayed the appearance of cirrhosis. Differences in the basal diets used for producing cirrhosis and in

TABLE I
EFFECTS OF VITAMIN B_{12} AND PENICILLIN ON THIOURACIL ACTION IN RHODE ISLAND RED CHICKS

Treatment[b]	Average body weight (gm.)		Food gain ratio	Average thyroid weight per 100 gm. body weight (mg.)
	Initial	Gain		
Controls	88	125.5	3.27	15.7
4μg. % B_{12}	86	140.1	3.08	17.5
T	88.7	97.1	4.07	153.4
T + 4 μg. % B_1	88.5	123.7	3.05	143.4
T + penicillin	76	137.3	2.81	138.4
T + 4 μg. % B_{12} + penicillin	75.2	154.2	2.59	159.0

Libby and Meites (1952).
[b] Twelve per group. T = thiouracil.

the resulting severity of choline and cystine deficiencies may be responsible for these conflicting reports.

b. Vitamin B_1. An increased intake of some vitamins may be beneficial to animals in the hypothyroid state. Thus, inhibition of body growth induced by thiouracil in immature rats on a vitamin B_{12} deficient diet was counteracted by feeding or injecting high doses of this vitamin (Meites, 1950a, Zarrow *et al.*, 1957). Thiouracil induced retardation of growth in chicks fed a vitamin B_{12} deficient diet was likewise prevented by feeding abundant vitamin B_{12} or small amounts of penicillin (Libby and Meites, 1952). This is shown in Table I. In both rats and chicks, food intake and efficiency of converting food into body weight gains were increased by the additional vitamin intake. Since no carcass analysis was made in these animals, the character of the growth increment is not known. Scow (1951)

demonstrated that forced feeding of thyroidectomized rats produced gains in body weight beyond that of thyroidectomized rats fed ad libitum, but 55 % of the carcass increment was fat and only 9 % was protein In thyroidectomized rats fed ad libitum the carcass weight gain was 32 % fat and 16 % protein, and in normal rats only 13 % fat with 21 % protein

It may be mentioned that protective effects of desiccated whole liver or meat meal for growth of rats treated with thiouracil or other goitrogenic agents have been reported (Ershoff, 1954, Ackerman, 1959) However, the question of iodine content and possible thyroidal activity in such materials was raised by Ershoff (1954)

c Dietary Effects on Thyroid Function Of the numerous reports on this problem only a few will be mentioned Food restriction in rats and mice reduced thyroid function, as judged by I^{131} uptake and thyroid weight, in accordance with the degree of underfeeding (Meites and Agrawala, 1949, Meites and Wolterink, 1950) Whether this effect is due to lack of calories or of protein is not known

Excess vitamin A has been reported by several workers to reduce metabolic rate (reviewed by Drill, 1943) Daily dosages of 30,000 I U vitamin A depressed basal metabolism and reduced thyroid weight in normal, thiouracil treated, and hyperthyroid rats (Sadhu and Brody 1947) These changes were accompanied by a decrease in liver and thyroid protein bound iodine (PBI) and an increase in serum PBI (Sadhu and Truscott, 1948) Whether these effects are due to depression of food intake or to interference with thyroxine utilization is not known This problem requires further investigation

3 Summary

In general, hyperthyroidism has been shown to increase the need for many dietary essentials, whereas hypothyroidism appears to ameliorate some dietary deficiencies Although these changes in dietary requirements are usually attributed to changes in basal metabolism, appetite, food intake, absorption and excretion, effects of the thyroid on other endocrine organs and the nervous system may also be involved The finding that vitamin B_{12} supplementation improves the growth of hypothyroid rats and chicks emphasizes the desirability of optimal nutrition for any condition resulting in decreased appetite and food intake

IV Adrenal Cortex

1 Administration of Glucocorticoids or ACTH

Large doses of glucocorticoids or ACTH increase protein catabolism and promote gluconeogenesis resulting in increased urinary nitrogen ex

of cortisone are known to increase urinary losses of vitamin B_1 in the rat (Chow, 1954, Ginoulhiac and Nani, 1956, Meites *et al*, 1957) and in the pig (Wahlstrom and Johnson, 1951) The urinary losses of vitamin B_1 appear to be more pronounced on vitamin B_1 deficient diets than on vitamin B_{12} supplemented diets (Table II)

b Thiamine Injections of 1 mg cortisone daily into young male rats fed a thiamine deficient diet aggravated their condition, as judged by reduced rate of growth and survival, and loss of muscular coordination Supplementation with 5 mg or more of thiamine per kilogram of diet, equivalent to several times the normal requirement, completely counteracted these detrimental effects of cortisone and permitted growth equiva

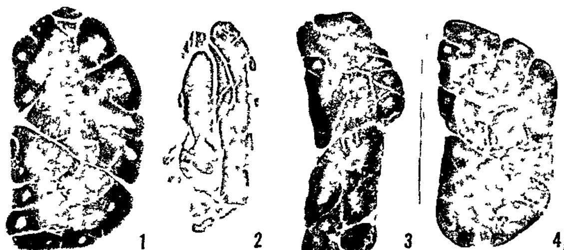


FIG 3 Histological appearance of representative thymus glands from rats shown in Fig 1 Treatment was as follows 1 vitamin B_1 2 cortisone and no vitamin B_1 3 cortisone and vitamin B_{12} 4 cortisone and Aureomycin From Meites (1952)

lent to that obtained in uninjected rats supplemented with 1 mg of thiamine (Wilwerth and Meites, 1953, Wilwerth, 1955, Meites *et al*, 1957) This is shown in Fig 4 The latter amount of thiamine represents the normal requirement for the growing rat (Brown and Sturtevant, 1949) When rats were given 1 mg cortisone daily and 20 mg of thiamine per kilogram of diet but were limited in food intake to that of thiamine deficient, cortisone treated rats, the supplemented rats failed to grow but survived and did not exhibit the characteristic symptoms mentioned above

Biochemical studies revealed that when 1 mg of cortisone was injected daily into thiamine deficient rats, there were smaller losses of nitrogen and less hyperglycemia than in thiamine supplemented rats also injected with cortisone This is in contrast to the effects of cortisone in vitamin B_1 deficient rats, in which urinary nitrogen losses and hyperglycemia were

better utilization of food may, therefore, account at least in part for th beneficial effects of additional thiamine in cortisone injected rats

In guinea pigs the injection of 5 or 10 mg of cortisone daily for 20 da did not increase weight losses on thiamine deficient diets (Wilwerth, 195 Meites *et al*, 1957) This is in agreement with the report of Hausberger ar Ramsay (1953) that large doses of cortisone do not induce loss of weigl in guinea pigs In thiamine deficient guinea pigs, cortisone induced neith hyperglycemia nor increased urinary nitrogen losses, whereas in thiamin supplemented guinea pigs cortisone induced hyperglycemia without si nificant change in urinary nitrogen excretion It is apparent, therefor that the interactions between cortisone and thiamine are not the same i the guinea pig as in the rat

It is of interest that large doses of adrenal glucocorticoids or ACTI have been reported to decrease urinary losses of thiamine in normal an in hypophysectomized rats (Ginoulhiac and Nani, 1956, Marchetti *et al* 1955), suggesting that more of this vitamin is being utilized within th body Rindi and co workers (1955) reported that injection of 1–4 mg c cortisone reduced the thiamine content of liver, brain, and muscle of rat on a normal diet, but not on a thiamine deficient diet An average decreas of 27% in urinary thiamine excretion was observed in ten children treate for various diseases with adrenocorticotropic hormone (ACTH) (Acet *et al*, 1956)

c Other B Vitamins Large doses of cortisone were found to inhibi body growth in young rats to a greater extent on diets deficient in ribo flavin or pyridoxine than on diets supplemented with several times th normal requirement of these factors (Wilwerth, 1954, Meites *et al*, 1955 1957) The increased vitamin supplementation resulted in increased fooc intake and efficiency of food utilization for growth As with thiamine ex cretion, injection of glucocorticoids or ACTH was reported to decrease urinary losses of riboflavin in adrenalectomized or hypophysectomized rats (Laszt and Dalla Torre, 1943, Marchetti *et al*, 1955) However, Draper and Johnson (1953) found no change in urinary excretion of ribo flavin or pyridoxine when rats deficient in these vitamins were injected with 2 5 mg cortisone daily Dhyse *et al* (1953) also noted that cortisone injections into adrenalectomized rats during a 7 hour period did not alter the liver content of these two vitamins

Morgan and Simms (1940) reported that adrenal cortical extract (ACE) resulted in the partial cure of graying in "filtrate factor" deficient rats whereas Mushett and Unna (1941) were unable to prevent graying or adrenal hemorrhages in pantothenic acid deficient rats with either ACE or DCA However, Schultz *et al* (1952) observed that cortisone prevented (or delayed) the development of adrenal pathology in pantothenic acid

deficient rats Large doses of prednisone decreased urinary losses of pantothenic acid in rats according to Ginoulhiac and Nani (1956) Further studies will be required to determine whether glucocorticoids can alter the requirements for pantothenic acid

Ershoff (1951b, 1953a) reported that oral feeding of 100–200 mg of cortisone per kilogram of diet retarded growth, induced alopecia, and reduced survival in rats fed a purified diet Supplementation with 10% whole liver counteracted these effects of cortisone, whereas the known B vitamins were ineffective This unknown factor in liver is presumably similar to the antithyrotoxic factor" (see Section III, 1, c) In mice, growth inhibition induced by feeding 25 mg of cortisone per kilogram of diet was not counteracted by 10% whole liver

d Ascorbic Acid and Vitamin A Most investigators have found that administration of ACTH or cortisone delayed the onset of severe scurvy, maintained higher body weights, and prolonged the survival of scorbutic guinea pigs, e g Hyman *et al* (1950), Herrick *et al* (1952), Schaffenburg *et al* (1950) The beneficial effects of cortisone in scorbutic guinea pigs may be due to direct effects on mesenchymal tissues or to increased food intake In human patients ACTH administration has been reported to induce signs of ascorbic acid deficiency and to increase the urinary excretion of ascorbic acid (Stefanini and Rosenthal, 1950, Beck *et al* 1950) In dogs and rats cortisone administration increased plasma or serum concentration of ascorbic acid (Booker *et al*, 1951, Bodansky and Money, 1954)

Clark and Colburn (1955) observed a rapid depletion of liver and kidney stores of vitamin A when 3 mg of cortisone was administered daily to normal or adrenalectomized rats on either a stock or a vitamin A deficient diet, whereas Bodansky and Money (1954) reported depression of kidney levels but not liver or plasma vitamin A levels when 1–2 mg of cortisone was given daily Bodansky and Markardt (1951) found decreased plasma and kidney levels of vitamin A when 15–30 mg of Reichstein's compound L was used None of these workers indicated that signs of vitamin A deficiency were produced by the hormones

2 *Adrenalectomy*

The increased requirement of the adrenalectomized rat for NaCl is well established In addition high intakes of certain B vitamins may improve growth and survival of the NaCl maintained adrenalectomized rat, particularly when diets low in these vitamins have been used prior to the operation For example, rats given a pantothenic acid deficient diet for one month before adrenalectomy, required as much as 4 mg of the vitamin daily (several times the normal requirement) for survival and growth

ary nitrogen excretion Thiamine deficiency in guinea pigs was not
entuated by cortisone administration and ascorbic acid deficiency was
arently ameliorated by glucocorticoids or ACTH These findings em
size species differences in response to hormones
drenalectomized rats like hypophysectomized animals, apparently
uire an optimal diet for maximum survival and growth In addition to
Cl, protein and additional B vitamins appear to be essential in the ab
e of the adrenals

V Gonadal Hormones

1 Estrogens

he multiplicity of effects resulting from exogenous estrogens permits
study of many nutrition estrogen interrelations Toxic manifestations
h as growth depression or mortality resulting from high dosage levels
estrogens in rodents may be partially corrected by vitamin supplemen
on The morphological changes resulting from estrogen dosage may be
uenced by deficiencies or excess of specific vitamins The effect of estro
s in stimulating growth of specific tissues may be expected to increase
requirements of nutrients needed for this rapid growth

Growth Depression in Rodents The depression of growth and food
ake and gonadal atrophy resulting from high doses of estrogens in rats
l mice has long been known (Zondek, 1936) Zondek (1936, 1941) as
bed atrophy of the gonads to depression of pituitary gonadotropin se
tion and decreased growth to depression of growth hormone secretion
smuch as injection of gonadotropic hormone prevented the gonadal
ophy and injection of growth hormone stimulated growth in the estro
ized rats Beneficial effects of growth hormone administration on growth
l food intake in such animals have been reported by some investigators,
Richards and Kueter (1941), but not by others who may have used
ufficient dosage levels, e g Gaarenstroom and Levie (1939) Inasmuch as
luction in growth and food intake with estrogen has been reported in
absence of the pituitary (Noble 1939), other pathways may also be
olved Meites (1949) found that retardation of growth in rats by di
ylstilbestrol, a synthetic estrogen, was proportional to the reduction
food and fluid intake The natural estrogens, estrone and estradiol, were
s effective in reducing growth and did not decrease food consumption
the dosage levels tested

Funk and Funk (1939) first showed that the effects of high doses of
rogens were less noticeable in rats maintained on complete diets than in
ose given inadequate diets Synthetic estrogens had greater effects than
tural estrogens, although this may have been due to differences in estro

genic activity. Chamelin and Funk (1943) found that liver extract decreased the mortality of rats given high doses (650 mg. per kilogram body weight) of diethylstilbestrol.

Ershoff and Deuel (1946) reported that 4.5% yeast counteracted the retardation in body growth and ovarian weight resulting from low levels of α-estradiol (5 mg. per kilogram of purified diet). When the dietary estradiol level was doubled, yeast was not effective but 10% desiccated whole liver (Ershoff and McWilliams, 1948) or 20% dried alfalfa (Ershoff

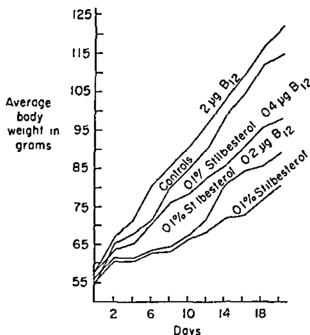


FIG. 5. Effects of diethylstilbestrol in diet and vitamin B_{12} injections on body growth of Carworth male weanling rats fed a diet considered adequate for normal growth. From Meites (1953).

et al., 1956) was markedly beneficial. The unidentified factor(s) in these supplements may be identical with the "antithyrotoxic factor" (see Section III, 1, c).

Meites and Shay (1951) found that even with massive doses of diethylstilbestrol (1 gm. per kilogram of diet) injections of 0.2–0.4 µg. vitamin B_{12} daily partially counteracted growth retardation in male rats and increased food consumption (Fig. 5). However, vitamin B_{12} did not prevent the marked depression in testis weight or the increase in seminal vesicle weight resulting from dietary diethylstilbestrol.

In contrast to the depression of growth in rodents by estrogen administration are the markedly anabolic effects of estrogens in ruminants. In cattle and in sheep estrogens stimulate growth, increase nitrogen retention,

and improve the efficiency of food utilization (e g Andrews, 1959) Struempler and Burroughs (1959) have reported that pituitary growth hormone content is increased by estrogen treatment of cattle, suggesting that the anabolic effects of estrogens in this species may be mediated through the pituitary In birds, estrogens increase food intake and fat deposition but have little or no effect on body weight (Lorenz, 1954)

b *Pteroylglutamic Acid (PGA or Folic Acid)* Pteroylglutamic acid has been shown to be essential for the action of estrogen in stimulating growth of the oviduct in chicks (Hertz and Sebrell, 1944, Hertz, 1945) and frogs (Goldsmith *et al*, 1948), inducing growth of the uterus (Hertz and Tullner, 1949a), involution of the prostate (Brendler, 1949), growth and development of mammary glands (Silver, 1954) in rats, and stimulation of the sexual skin, external genitalia, vagina, and uterine endometrium in monkeys (Hertz, 1948, Zarrow *et al*, 1950) The response of the chick oviduct and the rat uterus was shown to be directly proportional to the PGA content of the diet or inversely proportional to the severity of the deficiency (Hertz, 1945 Hertz and Tullner, 1949a) The beneficial effects of PGA supplementation on the response to estrogen of rats maintained on stock diets (Andreoli and Durando, 1953, Penhos, 1953) may probably be interpreted as evidence of previous suboptimal vitamin levels in such diets

The specificity of this inhibition of estrogen induced growth by PGA deficiency has been studied extensively in the chick Deficiencies of vitamin A, vitamin E, thiamine, riboflavin, pyridoxine, niacin pantothenic acid biotin, or choline did not impair the response to estrogen when oviduct weights were compared on a body weight basis or with pair fed controls (Kline *et al*, 1951) In fact, a slightly greater response than expected was observed for a few deficiencies, particularly that of thiamine However, vitamin B_{12} deficiency in the chick decreased the response to estrogen slightly but significantly (Kline, 1955) A purine analog 2 6 diaminopurine, likewise interfered with the response to estrogen in the chick, its effects were largely reversible by adenine but not by PGA (Hertz and Tullner, 1949b) The effects of this analog on response to estrogen in the rat have not been tested

The inhibitory effects of PGA deficiency on estrogen action in the chick, rat, frog and monkey can be reversed by PGA or folinic acid (LCF), depending on the PGA analog used Brown (1953) has reported a partial reversal by high levels of deoxyribonucleic acid (DNA) in the chick

It is not clear whether the mouse requires PGA for estrogen induced uterine growth Schoenbach *et al* (1950) reported that Aminopterin, even

[a] The restriction in food intake which accompanied vitamin deficiencies results in a nonspecific reduction in response of the chick oviduct to estrogen (Kline *et al* 1951)

when given at supralethal dosages to mice, did not affect the uterine weight response to estrogen On the other hand Velardo and Hisaw (1952) found that Aminopterin inhibited the local response of uterine endometrium and glands to estrogen, and Andreoli and Durando (1952) observed increased uterine weights in mice given estrogen when additional PGA was furnished

c Liver Inactivation of Estrogens The relation between rate of liver inactivation and nutritional status was first shown by Biskind and Biskind (1941) who observed that rats maintained on a vitamin B complex free diet lost the ability to inactivate intrasplenic implanted estrone pellets Addition of brewer's yeast restored the liver inactivation to normal (Biskind and Biskind, 1942) Segaloff and Segaloff (1944) and Singher *et al* (1944) found that both thiamine and riboflavin were necessary for this liver function but not vitamin B_6, pantothenic acid, biotin, or vitamin A

Further studies have brought out the importance of dietary protein Unna *et al* (1944) observed that rats maintained on an 8% casein diet failed to inactivate estrogens and maintain normal liver levels of riboflavin Methionine supplementation was beneficial in both respects Drill and Pfeiffer (1946) emphasized that inanition was primarily responsible for failure of estrogen inactivation by the liver and that this failure could not be reversed by methionine However, with better supplemented diets Jailer and Seaman (1950) and Vanderlinde and Westerfeld (1950) showed that protein intake rather than caloric intake was the critical factor in maintaining normal estrogen inactivation Recently Vasington *et al* (1958) have shown that *in vivo* addition of ascorbic acid to rats maintained on an 8% casein diet completly restored normal liver inactivation of estrogens, addition of glutathione was only partially successful *In vitro* addition of ascorbic acid to liver slices from such animals was ineffective

Diets producing fatty infiltration of the liver or hepatic necrosis resulted in decreased inactivation of estrogens before morphological changes were observed in the liver (Ferret, 1950) Choline (or methionine) and L cystine prevented both the loss of this function and the morphological changes in the liver observed later on such diets Vitamin E, on the other hand, was ineffective in restoring normal estrogen inactivation but prevented hepatic necrosis

The application of the above findings to clinical cases of disturbed hormone balance, as suggested by Biskind (1946) has been questioned Although liver inactivation of estrogens is rapid in experimental mammals, the rate is apparently low for primates, either man (Twombly and Taylor, Jr, 1942) or monkey (Hooker *et al*, 1947)

d Vitamin A and Vaginal Cornification The similarity of changes in the vaginal epithelium resulting from vitamin A deficiency and from estro

gen administration in ovariectomized rats have prompted many studies and much speculation on possible interrelations of these two substances The changes in vitamin A deficient epithelia were characterized by Wolbach and Howe (1925) as atrophy of epithelium accompanied or followed by proliferation of the basal cells which form a stratified keratinizing epithelium

Continuous vaginal cornification, considered to be the most sensitive indicator of vitamin A deficiency in rats (Aberle, 1933a, Mason and Ellison, 1935) occurs even after ovariectomy (Mason and Wolfe, 1935) The possibility of an increased sensitivity of vitamin A deficient rats to exogenous estrogen has been suggested but not proved However, it has been well established that excess vitamin A prevents the estrogen induced keratinization of the vaginal epithelium in rats and mice e g by Sherwood *et al* (1936) Thorborg (1948), Kahn and Bern (1950) When vitamin A and estrogen were applied topically to the rat vagina, as low a total dose as 0 6 I U of vitamin A significantly inhibited the vaginal cornification produced by 0 6 I U of estrogen given as α estradiol (Kahn 1954b) Moreover, a typical dose response relationship was observed The animals treated with both vitamin A and estrogen showed a stratified cuboidal epithelium rather than the typical estrogen induced cornification However, vitamin A did not inhibit estrogen induced hyperplasia of the epithelium Kahn (1954b) suggested that vitamin A may play a general role in inhibition of keratin formation, in addition to the specific effect of preventing the keratinization resulting from lack of the vitamin In tissue cultures of prepuberal vaginal epithelium, excess vitamin A suppressed keratinization occurring in control cultures and also delayed cornification when estrogen was added (Kahn 1954a)

2 Progesterone

The few studies on possible interrelations of nutritional factors with progesterone have been concerned, primarily with qualitative effects of this hormone, e g mucification of the vaginal mucosa and the characteristic progestational changes in the uterus

Vitamin A deficiency prevented mucification of the vaginal mucosa from injections of placental extracts (Aberle 1933b) or from circulating progestin during pregnancy (Mason and Ellison, 1935) However neither the size nor histologic characteristics of deciduomas induced in such animals were affected by vitamin A deficiency (Mason 1939)

PGA deficiency, induced by the antimetabolite Aminopterin inhibited the typical progestational reactions of the uterus resulting from exogenous progesterone in rats, mice, and rabbits (King and Velardo 1951 Velardo and Hisaw, 1952 1953), but not in monkeys (Zarrow *et al*, 1954) The

uterine response to progesterone of rats deficient in protein, potassium, thiamine, or vitamin B_6 was apparently normal, as shown by the maintenance of pregnancy in these four deficiencies with progesterone alone or in combination with estrone (Nelson, 1955, Nelson and Evans, 1954, 1955a,b, Nelson *et al*, 1951)

Although progesterone, like estrogens and other steroid hormones, is inactivated in the liver, no studies on the effect of nutritional factors on rate of inactivation have been reported

3 Androgens

Relatively few studies have been made on possible androgen nutritional interrelations Administration of testosterone propionate has been reported to accentuate deficiencies of biotin (Okey *et al*, 1950) and choline (Wilgram *et al*, 1956) in the rat This effect may be similar to that of growth hormone in increasing dietary requirements which depend on the rate of growth or on protein synthesis

PGA was not essential for stimulation of the male reproductive system by testosterone in several species, the response being reduced only in proportion to the accompanying inanition (Kline and Dorfman, 1951a) However, PGA has been shown to be essential for testosterone induced growth of the chick oviduct (Kline and Dorfman, 1951b) Vitamin A deficiency prevented the mucifying action of testosterone on the vaginal mucosa in rats (Burrill and Nelson, 1941), but did not affect response of the male reproductive system to this hormone (Mayer and Truant, 1949)

Liver inactivation of androgens was not decreased when rats were maintained on a B complex free diet (Biskind and Biskind, 1943), but was reduced to one half the control level when the diet was deficient in niacin and tryptophan (Bryson *et al*, 1950) Niacin was believed to be important because it is a constituent of diphosphopyridine nucleotide, a cofactor in one of the enzymatic reactions involved in testosterone metabolism (Sweat and Samuels, 1948) No confirmation of these studies has been reported nor have other dietary deficiencies been investigated for possible effects on androgen inactivation

Depression of growth and food intake by higher than physiological levels of testosterone propionate has been demonstrated (e g Kochakian and Endahl, 1959), but no studies on effects of nutritional supplements on this growth inhibition have been reported

4 Summary

Several interactions between gonadal hormones and nutrition appear to be well established Administration of excess estrogens to rodents apparently increased the need for vitamin B_{12} and unidentified factors, as these

supplements partially or wholly counteracted the inhibition of food intake and body growth in estrogenized rats. Hypertrophy of the female accessory sex organs in several species in response to physiological or high doses of either estrogens or androgens was inhibited in accordance with the severity of PGA deficiency. However, growth stimulation of the male accessory sex organs by androgens was unaffected by PGA deficiency.

Liver inactivation of estrogens is dependent upon adequate tissue levels of thiamine, riboflavin, choline, ascorbic acid, and protein. Hypervitaminosis A quantitatively inhibits estrogen induced keratinization of the vaginal epithelium, whereas vitamin A deficiency prevents mucification of this epithelium by progesterone or androgens.

VI. General Summary

This brief review of the literature has shown that an increased intake of vitamins or other dietary factors can enhance the ability of rats and other animals to withstand the effects of large doses of growth hormone, thyroactive substances, adrenal glucocorticoids, and estrogens. The effects of hypophysectomy, adrenalectomy, and perhaps thyroidectomy, may also be partially ameliorated by dietary supplementation. Hormonal imbalances have usually produced more detrimental effects on body growth, health, and survival of rats on vitamin deficient diets than on vitamin supplemented diets. The initial degree of growth inhibition induced by administering large doses of thyroactive substances, diethylstilbestrol, cortisone, growth hormone, or after removal of some endocrine glands, is apt to be severe and even after relatively long periods of time body weight may remain depressed. On a vitamin abundant diet, however, the initial inhibition of growth is often followed by a distinct upward trend which may reach the normal growth curve. Such observations indicate that the ability of young rats to adapt themselves to overdosage or deficiencies of some hormones depends, in part at least, on an increased intake of vitamins or other nutrients. In most cases, the increased vitamin intake is accompanied by greater food consumption and increased efficiency in utilizing food for growth and maintenance purposes. It should be noted, however, that when food intake is limited, abundant vitamin administration may be of little benefit to the animal.

The mechanisms whereby an increased vitamin intake benefits young animals in hormonally imbalanced states remain to be clarified. Large doses of thyroxine, growth hormone, or glucocorticoids may increase dietary requirements by increasing the rate of metabolism of carbohydrate, fat, or protein. Some anabolic processes may be inhibited. Larger than normal amounts of nutrients may be eliminated from the body, creating relative dietary deficiencies. It is also possible that increased amounts of vitamins

may be needed to help the body inactivate and eliminate the excess hormones from the body Some of the interactions between gonadal hormones and dietary factors, especially PGA and vitamin A, appear to involve local tissue requirements In states of marked hormonal deficiencies, an enhanced intake of vitamins or other nutrients may enable essential metabolic reactions to proceed at the highest rate possible in the absence of the hormones This is consistent with the widely accepted view that hormones do not initiate, but only control, the rate of reactions The greater need for vitamins during severe hormonal imbalances may also be related to the general problem of the effects of "stresses" on vitamin and other dietary requirements Vitamin B_{12} and the unidentified "antithyrotoxic factor" in liver appear to occupy a unique position in these respects, since they have been shown to be effective in more types of hormonal imbalances than other dietary factors studied thus far

Not all the effects elicited by hormonal imbalances are corrected following increased vitamin or other nutrient intake The increase in basal metabolism elicited by thyroactive substances, or decrease in adrenal weight induced by large doses of cortisone, do not appear to be influenced by an increased intake of nutrients Inhibition of hair growth by cortisone or reduction of thymus weight by large doses of thyroactive materials or cortisone are only partially counteracted by increased vitamin intake Although dietary supplementation appears to be generally helpful in states of hormonal imbalances, qualitative and quantitative dietary requirements for each type of hormonal imbalance have not yet been determined

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The Chemistry and Pharmacology of Angiotensin

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I SURVEY

Investigation of the role played by the kidneys in the pathogenesis of hypertension has revealed a biochemical mechanism, of uncertain physio

logical significance, for the release of extremely potent pressor agents ("over all constrictors") from blood plasma, chemically distinct from the adrenergic system in that these agents are peptides

In 1898 Tigerstedt and Bergman isolated from kidneys a proteinaceous material that enhanced the blood pressure on intravenous injection The material was named *renin* Forty years later it was shown by Braun Menendez *et al* (1939, 1943) in Argentina and, independently, by Page and Helmer (1940a, 1943) in the United States that renin acts as a proteolytic enzyme on a plasma protein which they called *hypertensinogen* or *renin substrate*, respectively The result of the interaction is the production of the already mentioned extremely active, heat stable and dialyzable peptide material, then called *hypertensin* or *angiotonin* Incubation with blood serum inactivated the hypertensive peptides, probably through the proteolytic action of enzymes not yet identified and usually designated as *hypertensinase* or *angiotensinase*

With time, the divergent nomenclature used by different workers became somewhat confusing To relieve the situation, representatives of the two

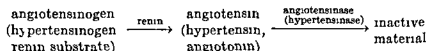


FIG 1 Genesis and destruction of angiotensin

groups agreed to the hybrid name *angiotensin* for hypertensin and angiotonin (Braun Menendez and Page, 1958) The other designations have, accordingly, been adapted, and the proposal has met quite general acceptance

The situation presented in the foregoing paragraphs is summarized in Fig 1

Subsequent work, especially that of Skeggs and his colleagues in Cleveland, has thrown much light on the chemistry of these conversions, as shown in Fig 2 Angiotensinogen contains a tetradecapeptide sequence from which the decapeptide *angiotensin I* (hypertensin I) is cleaved by the action of renin (the tetradecapeptide was isolated from the tryptic digest of angiotensinogen, it still acted as a substrate for renin and therefore was called *polypeptide renin substrate*) Angiotensin I is only slightly active on isolated arteries and other preparations *in vitro*, but it is active *in vivo* This is mainly due to the so called *converting enzyme* which cleaves off the *C* terminal dipeptide unit from angiotensin I, thus producing the octapeptide *angiotensin II* (hypertensin II) which is equally as active *in vitro* as *in vivo* (see Section VI, 2)

The work of Skeggs, which is summarized in Fig 2, was carried out with

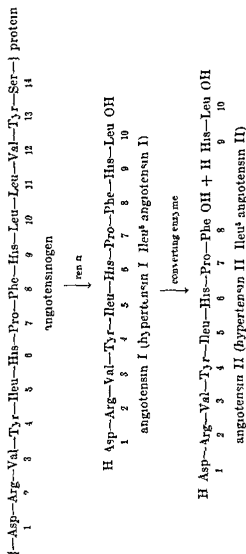


Fig 2 Biogenesis of angiotensin I and angiotensin II

angiotensinogen from the horse (using hog renin) According to Page and his group (cf Schwarz *et al*, 1957), angiotensin II produced from hog blood is possibly identical with that of the horse This is not the case with bovine angiotensin I, which had been isolated by Peart (1956a,b) Elliott and Peart (1956, 1957) found this decapeptide to differ from equine angiotensin I in that the residue of isoleucine in position 5 (counting amino acid residues from the *N* terminal) is replaced by that of valine This is summarized in Fig 3

Usually, the term angiotensin is used for material originating from any species To indicate precisely which compound is meant, Schwyzer *et al* (1957) have proposed the use of prefixes in connection with the terms angio tensin I or angiotensin II The prefix conveys information about the nature and position of certain amino acid residues in question The system is useful not only to differentiate between natural angiotensins, but also to designate structural analogs Thus, the equine octapeptide (Fig 3) would be called Ileu^{5} angiotensin II, and an analog of this peptide containing, say, glycine instead of phenylalanine would be named Ileu^{5} Gly^{8} angio tensin II

Syntheses of angiotensins have been carried out in Cleveland by the Page group and in Basle by the author's group The Americans have synthesised Ileu^{5} angiotensin II, the Swiss, in addition to this compound, have synthesized Val^{5} angiotensin I, Val^{5} angiotensin II (hitherto not shown to exist in nature), as well as a number of derivatives and analogs thereof (see Sections IV, 6 and V) Because only extremely small amounts of angiotensin (I and II) may be produced from blood by biochemical means, the synthesis of these compounds in appreciable quantities has given great stimulus to pharmacological and clinical investigations

The most obvious biological activity of angiotensin *II* (the activity hitherto studied most extensively and forming the basis for the assay procedures) is the sharp rise of blood pressure upon intravenous injection The blood pressure time diagram is very similar to that of norepinephrine (noradrenaline) and *is characterized by a sharp onset and short duration* of the effect, but the potency of angiotensin II is ten to twenty times greater It is not yet clear whether the action upon the blood vessels is the physiological function of the renin angiotensin system There is also a *strong action upon smooth* muscles (Braun Menendez, 1956) and the kidney (antidiuresis, cf Bock and co workers, 1958b), and it may well be that the physiological function (if it is really such and not pathological) is exerted within this latter organ (Gross, 1958)

Val^{5} angiotensin II Asp β amide (Fig 10, IIb) is clinically about ten times more active than norepinephrine and is being used in cases of shock and circulatory collapse It is effective even in very severe cases where

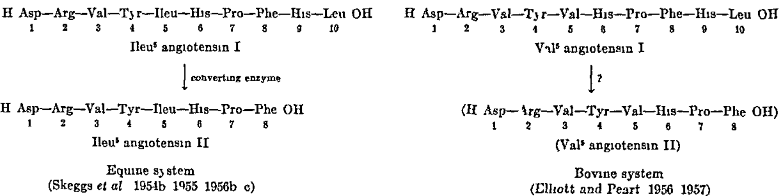


FIG 3 Equine and *bovine angiotensins* The presence of bovine converting enzyme and of Val[5] angiotensin II in nature has not *been demonstrated* Val[5] angiotensin II has been *obtained by synthesis*

treatment with norepinephrine is insufficient (Kipfer, 1959) The product is being marketed in various countries under the name Hypertensin CIBA®

A number of excellent reviews on the pharmacological and intricate historical aspects of the subject have appeared (Edman, 1945, Schales, 1947, Braun Menendez, 1956, Gross 1958) Therefore, this article is con cerned with some of the more recent chemical work only, advances in the pharmacology are treated in Section VI by Dr H Turrian

Other peptidic materials which raise blood pressure have also been de scribed Of these, only pepsitensin (Croxatto and Croxatto, 1942) and "substance A" (Walaszek and Huggins, 1959) shall be mentioned Pepsi tensin, arising from the action of pepsin on blood plasma proteins, seems to be chemically distinct from angiotensin (Serebrovskaya, 1954)

II ANGIOTENSINOGEN

1 *Historical Remarks*

In the year 1938, Kohlstaedt, Helmer, and Page (1938, 1940) demonstrated that, on perfusion of the dog's isolated tail, renin per se produces no vasoconstriction, but does this only in the presence of blood serum Very soon, independently of each other, two groups were able to show that contact of renin with blood serum produces heat stable vasoconstrictor substances (angiotonin Page and Helmer, 1940a,b, Page, 1940, hypertensin Braun Menendez *et al*, 1939) Subsequent communications and discussions by the two groups (cf Edman, 1945, Schales, 1947) did not completely clarify the relationship between renin and the "renin activator" present in serum [renin activator, hypertensinogen, renin substrate, prehypertensin, preangiotonin, and hypertensin precursor were the suggestive names pro posed at the time (cf Schales, 1947)] It was, however, demonstrated with reasonable accuracy that this substance (now called angiotensinogen) is located in the pseudoglobulin fraction (Munoz *et al*, 1940) and that it is associated with the α_2-globulins (Plentl *et al*, 1943), possessing similar electrophoretic mobility

Production of the heat stable vasoconstrictor agent angiotensin from the heat labile agents renin and angiotensinogen made an enzyme substrate relationship between the two seem highly probable (cf Edman, 1945, Schales, 1947) This was clearly proved by the investigations of Skeggs and his colleagues (1957, 1958), who demonstrated that renin acts as a proteolytic enzyme of a very unique type, cleaving the peptide bond between two leucine residues in angiotensinogen to produce angiotensin I (Fig 2) This uncommon specificity would seem to suggest that the production of angiotensin by renin is not fortuitous and pathological, but is part of a physiological process

Angiotensinogen is produced in the liver (Page and co workers, 1941) and is found in cervical lymph (Friedmann and co workers, 1943) as well as in the blood

2 *Isolation and Purification*

Fractionation of serum proteins to yield products enriched with angiotensinogen has usually been carried out by precipitation with ammonium sulfate In addition to the procedures mentioned in earlier reviews by Edman (1945) and Schales (1947), Green and Bumpus (1954) have developed a method for the purification of hog angiotensinogen by repeated precipitations at three different pH values (6 5, 4 0, and 6 0) with ammonium sulfate (between 1 2 and 2 25 *M*), followed by fractional precipitation at pH 2 5 with three levels of concentration of ammonium sulfate (1 44 1 64, and 2 05 *M*) The precipitates obtained were treated with renin, and the angiotensin produced was biologically assayed against a standard of low purity obtained at the beginning of the process A purification 6500 fold that of the standard was claimed for the precipitate at 1 64 *M* salt solution Electrophoresis showed that the purest preparations still contained contaminants (mainly γ globulin) and that the activity resides in the α_2-globulin fraction Angiotensinogen probably constitutes less than 0 1% of the proteins of serum

3 *Partial Structure of Angiotensinogen* (Skeggs and co workers, 1957)

a Crude Angiotensinogen 1020 liters of horse plasma were precipitated with ammonium sulfate in the range of 1 3 to 2 3 *M* according to Skeggs *et al* (1956b) Hypertensinase present in the preparation was destroyed by incubation of a 5% solution at pH 3 8 and 25°C for 30 minutes Precipitation between 1 4 and 2 2 *M* ammonium sulfate and dialysis yielded 2840 gm of crude angiotensinogen

b Preparation of "Polypeptide Renin Substrate" Tryptic digestion of angiotensinogen releases (in a very fast reaction) a polypeptide substrate for renin This may be assayed after the precipitation and removal of proteins by alcohol, by reaction with renin to produce angiotensin, and subsequent measurement of the pressor response in dogs or rats (Goldblatt, 1948) Owing to inactivation by trypsin in a slower reaction (cleavage of the Arg—Val bond in the tetradecapeptide Fig 2), the maximum yield of the polypeptide substrate is obtained after about ½ hour of incubation under the conditions employed

Along with the tetradecapeptide, an unknown pressor substance originates which is active per se and needs no incubation with renin to produce this property It is not known whether this product arises from the interaction of trypsin with another protein present in the preparation or with

another portion of the angiotensinogen molecule Generation from the tetradecapeptide by the action of traces of chymotrypsin or of carboxypeptidase seems less probable because the trypsin used was recrystallized material and the latter enzymes were inhibited by the addition of hydrocinnamic acid (Neurath and Schwert, 1950) The formation of the pressor agent is diminished by the addition of 20% of ethanol to the incubation mixture

The purification of the polypeptide material, soluble in 75% ethanol, to a stage where countercurrent distribution (CCD) gave distribution

TABLE I

PURIFICATION OF POLYPEPTIDE RENIN SUBSTRATE (2700 FOLD)

Step	Method	Units/mg N	Total units
0	Soluble in 75% ethanol	2 2	23 000
I	Removal of neutral product by precipitation with NaCl	19 3	21 400
II	Extraction with BuOH from pH 7 0 and re extraction into 0 01–0 03 N HCl	52	18 000
III	Adsorption from BuOH with Al_2O_3 extraction with boiling H_2O	183	15 750
IV	Countercurrent distribution 590 transfers $K = 1\ 02$ in *sec* BuOH/0 01 N $NaHCO_3$ – 0 01 N Na_2CO_3	720	15 000
V	Isoelectric precipitation pH 7 4	2200	14 200
VI	Countercurrent distribution 668 transfers $K = 2\ 76$ in *sec* BuOH/0 01 N HCl 2% NaCl	5900	10 800

[a] Goldblatt units (Goldblatt, 1948 Braun Menendez 1956)

curves for activity and nitrogen content which were similar to those predicted by theory for one component is shown in Table I Only 6 micromoles (?) were obtained from over 1000 liters of plasma

Paper chromatography in the solvent systems BuOH AcOH H_2O (4 1 5) and phenol water (100 39 5) gave single spots with R_f values of 0 57 and 0 87, respectively

c Amino Acid Sequence of Polypeptide Renin Substrate In addition to the amino acids present in angiotensin I, serine was detected by qualitative inspection of the total hydrolyzate Quantitative amino acid analysis by spectrophotometric evaluation of the dinitrophenyl amino acids prepared from the total hydrolyzate gave the following composition (Leu + Ileu)$_{3\ 11}$, Asp$_{1\ 02}$, Pro$_{0\ 85}$, Val$_{1\ 96}$, Tyr$_{2\ 26}$, Phe$_{1\ 03}$, His$_{1\ 87}$, Arg$_{1\ 13}$, and Ser$_{0\ 73}$ This result shows that the compound contains, above the amino

acid content of angiotensin I, one residue each of Leu (or Ileu), Tyr, Val, and Ser

These latter residues constitute one of the two peptides obtained by cleavage with hog renin one is angiotensin I, H Asp Arg Val Tyr Ileu His Pro Phe His Leu OH, the other is H Leu Val Tyr Ser OH (stepwise degradation with phenyl isothiocyanate) Separation was effected by paper chromatography The *N* terminal of the polypeptide renin substrate was shown to be Asp

Hence, the renin substrate obtained by tryptic digestion of horse angiotensinogen is the tetradecapeptide H Asp Arg Val Tyr Ileu His Pro Phe His Leu Leu Val Tyr Ser OH

d Synthesis of Polypeptide Renin Substrate The structure of the polypeptide renin substrate, deduced in the manner described, was further con firmed by synthesis (Skeggs and co workers, 1958) Like the product ob tained by tryptic digestion of angiotensinogen, the synthetic material was amenable to cleavage by porcine renin to give angiotensin I

The synthesis by Skeggs and associates (1958) is summarized in Fig 4 Two hexapeptide derivatives, *A 3–8* and *A 9–14*, were condensed by the azide method to give the dodecapeptide derivative *B 3–14* The carbo benzoxy group was removed by hydrogenolysis (the method of choice in this synthetic work), and the dipeptide unit *A 1–2* was then attached by the mixed anhydride method using isobutyl chloroformate (Vaughan, 1951) This is the only step in the whole synthesis of the tetradecapeptide applying the method of mixed anhydrides to the condensation of *peptide* derivatives with free carboxylic groups and thus endangering optical purity (here, of the residue of nitroarginine) In most of the other stages of the synthesis the safer azide procedure was used, and the mixed anhydride method was employed only when carbobenzoxy amino acids were condensed to the free amino group of amino acid or peptide derivatives, thus excluding partial racemization

The remainder of the synthesis, cleavage of the protecting groups by hydrogenolysis and mild alkaline hydrolysis, proceeded in the manner and sequence already chosen in the syntheses of angiotensin peptides by Schwyzer and co workers (1956, 1957, 1958a,b,c) and by Rittel *et al* (1957a,b), as discussed in Section IV, 6

Syntheses of the intermediate hexapeptide derivatives (*A 3–8*, *A 9–14*) are described in detail in Figs 5 and 6 The dipeptide derivative *A 1–2* (solid, m p 83–85°, $[\alpha]_D$ −3 0, *c* 5 24 in pyridine) was obtained by con densation of the mixed anhydride prepared from carbobenzoxy β benzyl L aspartate and isobutyl chloroformate with the sodium salt of nitro L arginine in a yield of approximately 48 %

e Discussion The specific ability of renin to cleave a leucyl—leucyl

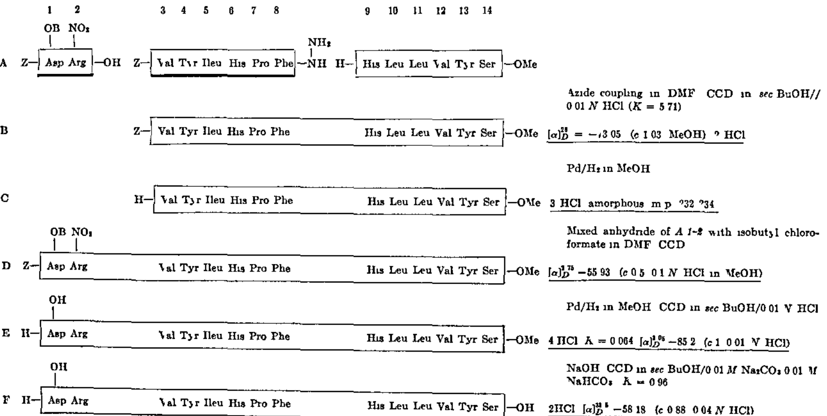


FIG 4 Synthesis of tetradecapeptide renin substrate Z— = C_6H_5—CH_2OCO— B— = $C_6H_5CH_2$— DMF = dimethylformamide CCD = countercurrent distribution heavy underline indicates crystalline intermediate Pd/H_2 = palladium catalyst hydrogenolysis c = concentration (per cent)

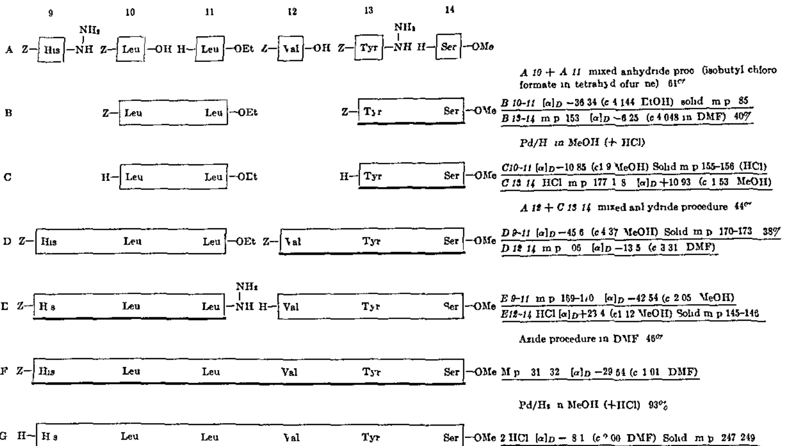


Fig 5 Synthesis of intermediate hexapeptide 9-14 of tetradecapeptide renin substrate (cf Fig 4 legend)

A Z—Val—OH H—Tyr—OEt Z—Ileu—OH Z—His—NH—NH_2 H—Pro—OB H—Phe—OMe

B Z—His Pro—OB

HCl solid m p 84-85 $[\alpha]_D$ −76 21 (c 1 77 H_2O) Yield 60%

hydrazine hydrate in MeOH room temp 1 week 61%

C Z—His Pro—NH—NH_2

? HCl solid m p 170 $[\alpha]_D$ −46 74 (c 3 13 MeOH)

Azide procedure in EtOAc 57%

D Z—His Pro Phe—OMe

Solid m p 87–89 $[\alpha]_D$ −41 59 (c 2 04 MeOH)

Pd/H_2 in MeOH HCl 95%

E H—His Pro Phe—OMe

2 HCl solid m p 145–147 $[\alpha]_D$ −18 94 (c 1 98 MeOH)

A3 + *A4* via mixed anhydride procedure (isobutyl chloro formate) 64%

F Z—Val Tyr—OEt Z—Ileu His Pro Phe—OMe

F3-4 m p 155–157 $[\alpha]_D$ +55 8 (c 1 01 $HCCl_3$)

A5 + *E6* 8 via mixed anhydride procedure (isobutyl chloroformate) 87%

G Z—Val Tyr—NH—NH_2 H—Ileu His Pro Phe—OMe

G3-4 m p 248–250 $[\alpha]_D$ −12 95 (c 4 25 DMF) yield quantitative

G 5-8 2HCl solid m p 100–102 $[\alpha]_D$ −58 92 (c 1 MeOH)

Azide procedure in EtOAc 74%

H Z—Val Tyr Ileu His Pro Phe—OMe

M p 184 $[\alpha]_D$ −68 29 (c 1 MeOH)

hydrazine hydrate in EtOH room temp 24 hr 85%

I Z—Val Tyr Ileu His Pro Phe—NH—NH_2

M p 177–178 $[\alpha]_D$ −82 18 (c 0 825 MeOH)

FIG 6 Synthesis of intermediate hexapeptide 3-8 of tetradecapeptide renin substrate (cf Fig 4 legend)

bond in angiotensinogen is one of the most remarkable results of the work described. No other proteolytic enzyme is known to display a similar specificity for peptide bonds between two aliphatic amino acid residues.

The question arises whether the lysis of this single peptide bond is the sole function of renin in the production of angiotensin. Cleavage of a peptide bond connecting the aspartic acid residue (No. 1, Fig. 2) to the rest of the molecule of angiotensinogen might also be envisaged. Skeggs *et al.* (1957) express the opinion that the residue of aspartic acid is *N* terminal and that the connection of the tetradecapeptide renin substrate with the rest of the molecule of angiotensinogen might proceed through an ester linkage, possibly involving the —OH of serine.

This seems quite reasonable as trypsin is known to attack ester bonds of synthetic substrates much more rapidly than the corresponding amide bonds (Schwert *et al.*, 1948). Such a kinetic preference, however slight, must be present to produce any of the tetradecapeptide at all, as the arginyl—valyl bond (2–3 in Fig. 2) is very readily cleaved by trypsin. Kinetic differences are hardly to be expected between this bond (Arg—Val) and a hypothetical R—Asp bond, so that one is justified in believing that Asp really is *N* terminal. Another consideration which is in favor of this hypothesis is one of probability: formulation of a peptide bond R—Asp would mean that renin as well as trypsin should display similar specificity as regards this particular bond, or that the renin preparation used contains as contaminant an enzyme that splits this bond but does not attack Arg—Val (angiotensin is stable to renin).

It is very remarkable that no pressure reducing substance was observed during the formation of the tetradecapeptide renin substrate by trypsin, as it is known that trypsin releases bradykinin (Rocha e Silva *et al.*, 1949) from an α_2 globulin (bradykininogen) very closely related to, or possibly even identical with angiotensinogen. This might of course be explained by the specific test methods used in the work of Skeggs and co-workers (1957), by destruction of bradykinin by renin, or by assuming that the angiotensinogen used was free of bradykininogen during the process of isolation (cf. Werle *et al.*, 1950).

III. Renin

1. Introduction

Renin, associated with proteins, or itself a protein, is found not only in the kidneys (Tigerstedt and Bergmann, 1898) but seems to occur also in the adrenals and in the hypophysis (Granzer, 1952). It is thermolabile, being destroyed by heating to 56°C. Incubation in acidic solution (pH 4 at 25° or pH 1.6 at 0°) does not inactivate renin, but does destroy angioten-

sinase (Fig 1) This reaction constitutes part of the procedure for the detection of small quantities of renin, e g as developed by Fasciolo and Taquini (1947), and is usually employed in the purification of renin (Haas *et al*, 1954) Recently, Cook and Pickering (1959) were able to demonstrate that renin is most probably associated with the juxtaglomerular cells in the kidney

In contrast to angiotensin, the response of blood pressure to the intravenous injection of renin is characterized by slower increase and (especially in nephrectomized animals) longer duration If the pressure increase is in this case due to release of angiotensin from plasma by renin, one should expect renin to be only very slowly altered in presence of whole blood, liberating angiotensin during the whole time of enhanced pressure It has not been proved that the renin isolated is the native enzyme that occurs in kidney tissue and is not a product of partial degradation or denaturation (Haas *et al*, 1953a) Nor can physiological actions of renin other than those elicited via angiotensin be excluded (Gross, 1958) Renol, a pressor substance from the kidneys (Stepun *et al*, 1955), has been shown to be identical with renin (Haas and Goldblatt, 1958)

Our knowledge of the chemistry of renin is less than our knowledge of its biochemistry and physiology Many attempts have been made to purify the enzyme, but none have led to complete success

2 Assay

The methods now in use for the assay of renin may be subdivided into direct methods relating the pressor response to the amount of renin injected, and indirect methods assaying the amount of angiotensin produced or the amount of angiotensinogen destroyed by the renin preparation (Braun-Menendez, 1956, Gross, 1958) As the interaction of renin with angiotensinogen is species specific, the results obtained may be subject to errors when the renin of one species is assayed with animals or angiotensinogen from other species The soundest approach from a chemist's point of view would certainly be that of reacting renin and angiotensinogen of the same species under strictly defined conditions (concentrations, other substances present, pH, etc) and determining the kinetics of the release of angiotensin

3 Isolation and Purification

Haas *et al* (1953b) have made a comparison of a number of previous methods for the isolation of renin and have developed a process for the production on a pilot plant scale of large amounts of highly purified renin from porcine kidneys The purification is about 56,000 fold, amounting to 780 units of renin per milligram of dry substance (for a comparison of the various standards in use see Braun Menendez, 1956) The preparation

was about forty times as active as the most active ones previously The procedure is based upon a process of autolysis to liberate renin and to destroy substances interfering with the later steps These involved fractionation by salts (e g with sodium tungstate, cf Astrup and Birch Andersen, 1947), with ethanol and acetone, and precipitation with HCl

Seasonal variations of the content of renin in the kidneys were observed maximum content is reached in springtime, in autumn and winter a decrease of three to fourfold was noted, the total amount of protein staying, however, at a constant level These variations might reflect the different age of the animals brought to the slaughterhouse during the different seasons

There seems also to be quite a difference in the content of renin in the kidneys of various species Again, the amount of protein extractable from a certain amount of tissue remains almost constant, but the amount of renin differs from 0 02 units of renin per gram of kidney (*Macaca mulatta* monkey) up to 12 units per gram (rabbit) Other species (rat, dog, human) show a rather low level of renin

These species differences were observed by Haas *et al* (1954) during the elaboration of a simple method for the extraction of renin Instead of the autolysis described in the previous paper (Haas *et al* 1953b), a sequence of freezing and thawing is used to liberate maximum amounts of renin Acidification of the extract to pH 1 6 with sulfuric acid removes about 85 % of the inert proteins Simple fractionation with salt results in an over all purification of thirtyfold This process might prove to be a convenient starting point for further work on the isolation and (extremely welcome) complete purification of renin

IV ANGIOTENSIN

1 *Introduction*

Earlier chemical work on the peptidic material arising from the action of renin on angiotensinogen, at that time named hypertensin or angiotonin, is described in the thesis of Pehr Edman (1945) During the subsequent nine years a great number of attempts completely to purify "hypertensin" were undertaken None of them led to the isolation of a pure compound, but much valuable experience was gained

Such experiments (see Section IV, 2) might have continued some time, had it not been for the observation of Skeggs and his co workers (1954a) that the materials hitherto examined consisted of a mixture of two closely related peptides Both of these, hypertensin I and hypertensin II (angiotensin I and angiotensin II) raise the blood pressure of animals when injected into the blood stream, but only one of them (angiotensin II) is

capable of causing a constriction of isolated vessels (Skeggs *et al*, 1956a) and aortic strips (Helmer, 1956, Helmer and Sanders, 1957) or of eliciting an oxytocic action on the isolated uterus of the rat (Bumpus *et al*, 1956)

Skeggs and his associates (1954a, 1956a,b) further observed that angiotensin II is produced from angiotensin I through the action of a peptidase present in plasma The enzyme (which is strongly activated by Cl^-) was called "hypertensin converting enzyme" (see Fig 2) It is most probably identical with "angiotonin activator" previously observed by Page and Helmer (1940b)

This work of Skeggs and his colleagues paved the way for further rapid progress Skeggs' group, working with renin from hogs and angiotensinogen from the horse, was able to isolate the two peptides in pure form and to determine their amino acid composition, as well as the sequence of the amino acid residues (Ileu[5] angiotensins I and II, Figs 7 and 8) (Skeggs *et al*, 1954b, 1955, 1956b,c, Lentz *et al*, 1956) Moreover, they were able to demonstrate that the converting enzyme is very specific and cleaves Ileu[5] angiotensin I between Phe (8) and His (9), producing Ileu[5] angiotensin II and the dipeptide histidylleucine (Lentz *et al* 1956)

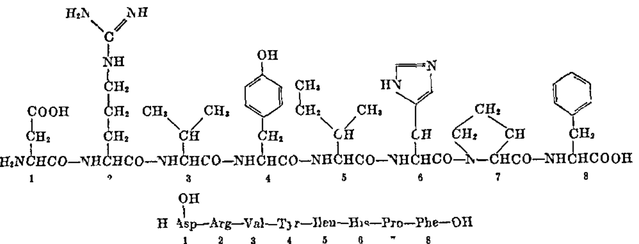
Peart (1955, 1956a), working with the blood of oxen treated with renin from rabbits, was able to do away with the destructive effect of peptidolytic enzymes (e g angiotensinase) and of the "converting enzyme" by the simultaneous addition of charcoal to the incubation mixture Angiotensin I was adsorbed onto the charcoal as soon as it was produced and was thus spared from the enzymatic action Subsequently, Peart (1956b) and Elliott and Peart (1956, 1957) were able to elucidate the structure of bovine angiotensin I as Val[5] angiotensin I (Fig 9)

Synthetic work was soon begun Active decapeptide material (Val[5] angiotensin I, Fig 10, IIIa,b) was obtained already during the summer of 1956 (Schwyzer *et al*, 1956) The group of Page (Bumpus *et al*, 1957, 1958, Page *et al*, 1957, Schwarz *et al*, 1957) synthesized Ileu[5] angiotensin II (Fig 10, Ia), whereas the CIBA group (in Switzerland) synthesized the amide (Asp β amide, Fig 10, Ib) of this compound (Rittel *et al*, 1957a,b) and the Asp β amides and dicarboxylic acids of (the known) Val[5] angiotensin I, and of the unknown, but expected, Val[5] angiotensin II (Schwyzer *et al*, 1957, 1958a,b,c, Fig 10 IIa,b, IIIa,b) A number of structural analogs of the peptides were also prepared and assayed (Schwyzer *et al*, 1957, Schwyzer, 1959) (cf Section V) Elliott and Russell (1957) made a preliminary report on a very interesting synthetic approach to intermediates of Val[5] *angiotensin I, using active esters*

Synthesis via the Asp β amides yields the corresponding octa and decapeptides in a very pure state, owing to the unexpectedly "benign" physical and chemical properties of the specific intermediates Thus, Val[5]

OH
H Asp—Arg—Val—Tyr—Ileu—His—Pro—Phe—His—Leu OH
1 2 3 4 5 6 7 8 9 10

FIG 7 Ileu[5] angioten in I



OH
H Asp—Arg—Val—Tyr—Ileu—His—Pro—Phe—OH
1 2 3 4 5 6 7 8

FIG 8 Ileu[5] angiotensin II

$H_2NCHCO—NHCHCO—NHCHCO—NHCHCO—NHCHCO—NHCHCO—N—CHCO—NHCHCO—NHCHCO—NHCHCOOH$

1 2 3 4 5 6 7 8 9 10

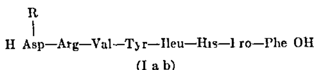
H Asp—Arg—Val—Tyr—Val—His—Pro—Phe—His—Leu OH
1 2 3 4 5 6 7 8 9 10

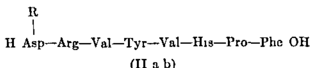
FIG 9 Val[5] angiotensin I

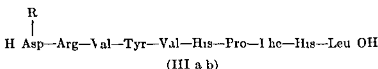
hypertensin II Asp β amide (Hypertensin CIBA) may be produced in large quantities and in a highly purified form

2 Procedures for the Isolation of Crude Mixtures of Angiotensin Peptides

Edman (1945) describes the purification of "angiotensin" obtained by the interaction of porcine renin with equine angiotensinogen Precipitation of proteins by the addition of alcohol to about 85–90% concentration, chromatography on Al_2O_3, precipitation of active material with nitranilic







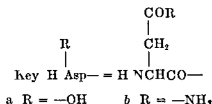


FIG 10 Synthetic angiotensin peptides

acid and electrodialysis resulted in a 600–700 fold increase of activity with a yield of only 3% This low yield was ascribed to 'extreme lability of hypertensin at higher levels of purification " Physical and chemical properties of synthetic material show that both angiotensin I and angiotensin II are very stable compounds and that the inactivation observed by Edman must be ascribed to the presence of other compounds (possibly catalyzing oxidation)

Later workers used paper chromatography (Helmer, 1950, Prado *et al* 1953) dialysis (Kahn *et al* 1950, Skeggs *et al* 1951), precipitation of proteins with ethanol, extraction into *n* butanol, adsorption onto Al_2O_3 and elution with mixtures of ethanol and water (Skeggs *et al* 1952), ion exchange and partition chromatography on Celite (Bumpus *et al*, 1954),

extraction with *n* butanol and precipitation therefrom with ether (Clark *et al*, 1954), and extraction with methanol and phenol, followed by partition chromatography, countercurrent distribution, and electrophoresis (Kuether and Haney, 1955)

All these procedures yielded only impure preparations The actual chemical nature as peptidic material was demonstrated by a number of the workers Especially, inactivation of the extracts by trypsin and other proteolytic enzymes served this purpose (Kahn *et al*, 1950, Skeggs *et al* 1951)

3 Differentiation and Separation of Angiotensin Peptides

It has already been mentioned that Skeggs *et al* (1954a) were able to demonstrate that two materials which are active *in vivo* exist in preparations of angiotensin Countercurrent distribution of crude angiotensin (produced from horse angiotensinogen with hog renin) in a solvent system composed of *n* butanol and 0 05 *N* sodium phosphate buffer (pH 7 0) showed a neat separation of two compounds with hypertensive activity (*in vivo*) After fifty transfers "hypertensin I" showed up in tubes nos 16–30 (maximum at 20), "hypertensin II" in tubes nos 4–12 (maximum at 6) It was demonstrated that the faster moving material (hypertensin I) yielded slower moving material (hypertensin II) on incubation with *crude* "plasma substrate" (angiotensinogen) in the presence of 0 1 *M* NaCl The enzyme present in the preparation of angiotensinogen, and responsible for the conversion, is not only activated by Cl^-, but also by NO_3^-, Br^-, and F^-, whereas HCO_3^-, SO_4^{--}, and HPO_4^{--} are ineffective "Hypertensin converting enzyme" was the name proposed, this would be "angiotensin converting enzyme" according to the new nomenclature The material was obtained in a semipurified form by fractionation with ammonium sulfate, dialysis, and isoelectric precipitation at pH 5 2 (Skeggs *et al*, 1956a)

With this enzyme preparation at hand, the authors were able to demonstrate conclusively that both angiotensin I and II elicit a hypertensive effect in the intact animal, but that only angiotensin II shows vasoconstrictor properties on perfusion of isolated rat kidneys (resulting in a large and sustained increase of perfusion pressure)

Subsequently, other authors were also able to demonstrate the existence of angiotensin in two forms Helmer (1956, 1957) produced evidence that patients with hypertension have a greater amount of converting enzyme present in plasma than normotensive subjects He used spiral strips of the aorta of the rabbit to differentiate between angiotensin I and II II produces an immediate response (contraction), I, only after conversion to II

Bumpus *et al* (1956) also used countercurrent distribution to separate the two angiotensins, using pressure response (*in vivo*) and oxytocic action

(*in vitro*) as tests They found the solubilities of the two to be very similar in acidic media but quite different in basic media, indicating that the iso electric point of angiotensin II (their oxytocic and pressor material) is lower than that of angiotensin I (their exclusively *in vivo* pressor material) This is in complete agreement with later findings on pure compounds

4 Isolation and Purification of Angiotensin Peptides

a Ileu[5] *Angiotensin I* Skeggs *et al* (1954b) isolated angiotensin I in the following manner

Angiotensinogen that had been obtained from citrated horse plasma by fractionation with ammonium sulfate (cf Section II, 2) and that had been very thoroughly dialyzed (removal of Cl^-) was incubated with hog renin until a maximal yield of hypertensin was attained At this point, ethanol was added to precipitate proteins, the ethanol was removed by distillation, and the aqueous residue defatted with ether, adjusted to pH 2 0, and cen trifuged The supernatant containing the activity was saturated with NaCl to precipitate active material This was dissolved in dilute HCl adjusted to pH 7 0, and extracted first into butanol, then with 0 15 N HCl, and again with butanol From this, the active material was adsorbed onto a column of alumina, and (after washing with 85% ethanol) eluted there from with boiling water The aqueous phase, after removal of $Al(OH)_3$, was concentrated to a small volume, and the active material was precipi tated by adjustment to pH 2 0 This was subjected to countercurrent dis tribution in the system *sec* butanol 0 01 N $NaHCO_3$ Na CO_3 buffer, pH 10 0 The method of single withdrawal was applied (106 tubes, 374 trans fers), the activity emerging in a peak with a distribution coefficient K = 0 6 The high pH of 10 0 was used to prevent adsorption, onto the glass surface, of the extremely small quantities of angiotensin present The pooled material was precipitated from aqueous solution, pH 7 0, by satu ration with NaCl, filtration, and washing with small amounts of water, eth anol, and ether

The material thus obtained contained 7050 Goldblatt units per milligram of nitrogen or 1125 units per milligram of solid As synthetic Val[5] angio tensin II Asp β amide (Fig 10, IIb), which per se is about twice as active as the decapeptide, was assayed as having 3200–3500 Goldblatt units per milligram of solid (through the courtesy of Dr S Deodhar, Mount Sinai Hospital of Cleveland, Ohio), it is probable that the natural hypertensin I prepared by Skeggs and his colleagues was about 70% pure on a weight basis

The impurities, however, do not seem to have been very disturbing, as preliminary hydrolysis experiments gave the expected nine amino acids Asp, Tyr, His, Arg, Pro, Val, Leu, Ileu, and Phe

b Val[5] Angiotensin I This decapeptide was prepared by Peart (1955, 1956a) from ox blood serum treated with rabbit renin and charcoal

Ox blood serum was treated with animal charcoal (7 gm per liter), and the charcoal discarded The residual serum was incubated with the renin preparation and the same amount of charcoal for 30 minutes The charcoal was separated by centrifugation, washed with water (addition of filter aid), and filtered After washing with ethanol, the active component was eluted with glacial acetic acid After evaporation of the solvent, the residue was dissolved in water and filtered clear, the solution was stored at 2°C

Judging from the assay data (pressor response of rats treated with ure thane and pentapyrrolidinium tartrate), one can now say that the product (100–200 mg per liter of serum) contained approximately 1 part per thou sand of angiotensin I

Final purification was achieved by partition chromatography in the systems butanol acetic acid water (9 1 10, $K = 0\ 1$) with Hyflo as carrier for the lower phase and butanol 0 25% trichloroacetic acid/water (1 4, $K = 15$) with siliconated Hyflo as carrier for the upper phase Three active components were separated The major one (purification 25 times and 5 times, respectively, content of hypertensin about 10–15%) was finally purified by adsorption on Hyflo from the aqueous phase Displacement with 0 02 *M* ammonium formate (pH 6 5) gave a highly purified component traveling at the front

The material was isolated by evaporation and drying at 30° and 0 01 Torr It showed an activity of about 1 5–3 times that of (−) norepineph rine in the assay described, *and may have had a purity of between 30 and* 60% [Ileu[5] angiotensin II-Asp β amide (Fig 10, Ib) (Rittel *et al*, 1957b) activity is five to ten times that of norepinephrine, but the octapeptide *per se may be more active by a factor of 2 in this test than the decapeptide*]

The isoelectric point of the material was found to be somewhere between pH 7 and 8 5 (synthetic Val[5] angiotensin I has pH 7 4, Schwyzer *et al*, 1958a) Treatment with fluorodinitrobenzene indicated only aspartic acid (or asparagine) to be *N* terminal Qualitative and quantitative amino acid analysis (paper chromatography of hydrolyzate followed by elution and photometry of the colored material produced by ninhydrin) showed eight amino acids to be present as ten residues 2 His Arg Asp, Pro, 2 Val, Leu, Phe, Tyr Ileu was absent in the hydrolyzate, and Try was missing in the peptide (ultraviolet absorption revealed only Phe and Tyr)

3 Ileu[5]-Angiotensin II Skeggs and co workers prepared crude Ileu[5] angiotensin II from purified Ileu[5] angiotensin I (Skeggs *et al*, 1954b) by incubation with purified and lyophilized converting enzyme for 4 5 hours at 37° (Skeggs *et al*, 1956b) After further purification *as described below*, 75 mg of Ileu[5] angiotensin II were obtained from 1375 liters of horse plasma

and 300 pounds of hog kidney The preparation was active at a level of 13,700 Goldblatt units per milligram of nitrogen [synthetic Val[5] angiotensin II Asp β amide (Fig 10, IIb) (Schwyzer *et al*, 1958b) contained approximately 26,000 Goldblatt units per milligram nitrogen]

Ileu[5] Angiotensin I was prepared according to Skeggs *et al* (1954b) with slight deviations from the original procedure Phenyl mercuric chloride was added to the blood in the preparation of angiotensinogen, and all operations were carried out in the cold to prevent gross bacterial contamination Renin was prepared in such a manner as to exclude the step of dialysis (which may be regarded as dangerous because of the possibility of infection) and not to introduce chloride ions (to keep the activity of converting enzyme low)

Conversion to Ileu[5] angiotensin II by means of enriched converting enzyme was carried out after sterilization of the solution of Ileu[5] angiotensin I by heating After incubation at 37°C for 4 5 hours, the reaction was terminated by addition of 4 volumes of 95% ethanol After adjustment of the pH to 5 2, Celite was added and the preparation was filtered The filtrate was evaporated to a small volume and extracted with ether Active material was precipitated at pH 2 0 by saturation with NaCl The precipitate which showed an activity of 9900 Goldblatt units per milligram nitrogen was purified by countercurrent distribution as described for Ileu[5] angiotensin I A major peak with $K = 0\ 24$ contained Ileu[5] angiotensin II (13 700 Goldblatt units per milligram nitrogen) and was clearly separated from unconverted Ileu[5] angiotensin I ($K = 0\ 55$)

5 *Structural Work*

a Amino Acid Composition of Various Preparations of Angiotensin A number of hypertensive preparations have been analyzed and their amino acid composition reported The results of such investigations are listed in Table II Early analyses (nos 1 2, 5) reflect the impurity of the products under consideration and help to demonstrate the great difficulties usually encountered in work on the isolation of more or less complicated peptides

As confirmed by later sequential analyses and by synthesis only the groups of Skeggs (Table II nos 3, 4, 6) and of Peart (Table II no 7) had material of such purity as to allow the determination of the correct amino acid composition Skeggs *et al* (1954b 1955, nos 3, 4) found angiotensin I from the horse to contain nine different amino acid the residue of histidine occurring twice Bovine angiotensin I, on the other hand (Peart, 1956a b, no 7), contains only eight different amino acid residues, the isoleucine being replaced by a second residue of valine This detail reveals a species difference in the angiotensins from the two different sources Angio

tensin II differs from angiotensin I by the deficiency of one residue each of histidine and leucine (Lentz *et al*, 1956)

The other authors listed in Table II seem to have hydrolyzed material contaminated by other peptides or amino acids Kuether and Haney (1955) claimed to have used an extremely potent preparation (although it did lose potency on standing, and that down to a very low value), but found altogether fifteen different amino acid residues These authors were not

TABLE II

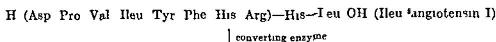
AMINO ACID COMPOSITION OF VARIOUS PREPARATIONS OF ANGIOTENSIN

No	References	Amino acids in total hydrolyzate
1	Edman 1945	Ala Asp Glu, Gly, His Leu (or Ileu) Lys Pro Tyr, Val
2	Bumpus and Page 1954	Ala_1, Arg_2 Asp_3 Glu_1, Gly_1, His_2 $Ileu_1$ Leu_2 Lys_1, Phe_1, Pro_2, Ser_1 Tyr_1 Val_1 *N* terminal Asp, *C* terminal Leu or Ileu
3	Skeggs *et al* 1954b	Arg Asp, His, Ileu, Leu Phe Pro Tyr, Val
4	Skeggs *et al* 1955	Arg_1 Asp_1 His_2 $Ileu_1$ Leu_1 Phe_1 Pro_1 Tyr_1 Val_1
5	Kuether and Haney 1955	Ala Arg Asp Glu Gly His, Ileu Leu, Lys Phe Pro Ser Thr Tyr, Val
6	Lentz *et al* 1956	Arg_1 Asp_1 His_1 $Ileu_1$ Phe_1 Pro_1 Tyr_1 Val_1
7	Peart 1956a b	Arg_1 Asp_1 His_2 Leu_1 Phe_1, Pro_1 Tyr_1 Val_2 *N* terminal Asp

able to confirm the first correct assignment of the *N* terminal (aspartic, Bumpus and Page, 1954), but found free amino groups only in the side chain of lysine The actual meaning of these findings is not at all clear Bumpus and Page (1954) were also able to consign leucine or isoleucine to the *C* terminal position This means that they were dealing with a prepara tion consisting mainly of angiotensin I

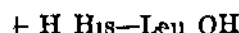
b Amino Acid Sequences of $Ileu^5$ Angiotensins I and II For the structural elucidation of the $Ileu^5$ angiotensins I and II (Skeggs *et al*, 1956c), their chemical relationship (Lentz *et al*, 1956) was of greatest importance $Ileu^5$ angiotensin I, with the amino acid composition Asp_1, Pro_1, Val_1, Leu_1, $Ileu_1$, Tyr_1, Phe_1, His_2, Arg_1, is converted to $Ileu^5$ angiotensin II,

Asp_1, Pro_1, Val_1, $Ileu_1$, Tyr_1, Phe_1, His_1, Arg_1, lacking histidine and leucine, by the action of angiotensin converting enzyme Inspection of the mother liquors (remaining after salting out of Ileu[5] hypertensin II) by extraction with butanol at pH = 1 4 and countercurrent distribution revealed the presence of histidylleucine as the sole ninhydrin positive material besides a small amount of angiotensin Its structure was proved by (1) total hydrolysis, yielding histidine and leucine, and (2) reaction with 1 fluoro 2,4 dinitrobenzene (FDNB), followed by hydrolysis of the dinitro phenyl (DNP) peptide to bis DNP histidine and leucine The relationship of the two could hence be formulated as follows


H (Asp Pro Val Ileu Tyr Phe His Arg)—His—Leu OH (Ileu[5] angiotensin I)

converting enzyme


H (Asp Pro Val Ileu Tyr Phe His Arg) OH (Ileu[5] angiotensin II)


+ H His—Leu OH

The sequence —His—Leu OH at the *C* terminal was confirmed by the action of carboxypeptidase and extended by one amino acid residue This enzyme released (1) leucine, (2) histidine and (3) phenylalanine The action then came to a stop, which, considering the specificity of carboxypeptidase, suggests that phenylalanine is attached to a proline or arginine residue The structure of the decapeptide might then be formulated as


H (Asp Val Ileu Tyr His Arg Pro)—Phe—His—Leu OH

Interesting changes in the pressor activity of the incubation mixture with carboxypeptidase were found to accompany the degradation from the *C* terminal Activity drops to a minimum as leucine is cleaved, possibly because the nonapeptide with *C* terminal histidine is not amenable to conversion to the octapeptide by the converting enzyme of the test animal With further release of histidine by carboxypeptidase, the activity rises again, only to drop to almost zero after cleavage of phenylalanine (cf Fig 11)

The complete structure was inferred from degradative work on the octapeptide and on the decapeptide, as well as the heptapeptide H (Asp Val, Ileu Tyr,His Arg Pro) OH resulting from the action of carboxypeptidase

The action of chymotrypsin on the octapeptide gave two peptides (cf Fig 11)


H (Asp Val Tyr Arg) OH (A)


H (Pro Ileu, Phe His) OH (B)

Because of its content of phenylalanine, (B) must represent the *C* terminal half of Ileu[5] angiotensin and, according to the results of the degradation

by carboxypeptidase, as well as the determination of the sequence by Edman's phenylthiohydantoin procedure (three steps), it may be conclusively represented by



Peptide A, accordingly, represents the *N* terminal half of the octapeptide. Because of the specificity of chymotrypsin, it may be written H (Asp, Val, Arg) Tyr OH. Further work was done on the more readily available decapeptide after it had been shown that it possesses *N* terminal aspartic acid in common with the octapeptide. Edman's procedure, and Sanger's DNP-method, used in combination, revealed the sequence H Asp—Arg—Val ; tyrosine could not be definitely located.

This last task was done by *partial acid hydrolysis* of the heptapeptide

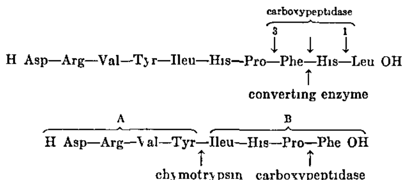


FIG. 11. Enzymatic cleavage of Ileu[5] angiotensins I and II.

obtained by degradation with carboxypeptidase. Partial acid hydrolysis with 3 *N* HCl at 100°C during 3 hours yielded, among others, the tripeptide H Arg—Val—Tyr OH, which could be isolated using a Dowex 50 X2 column. Its sequence was unequivocally proved by Edman's degradation (PTIC). This places tyrosine next to valine.

The structures shown in Figs. 7 and 8 follow unequivocally from the series of reactions described and from the fact that no ammonia was liberated on total hydrolysis. The various enzymatic cleavages used are summarized in Fig. 11.

c. Amino Acid Sequence of Val[5] Angiotensin I. The elucidation of the structure of this decapeptide (Fig. 9) was performed by Elliott and Peart (1956, 1957); it is based on the determination of the amino acid composition by Peart (1956b).

Action of leucine aminopeptidase (probably containing prolidase) released all the amino acids constituting Val[5] angiotensin I in increasing amounts during the time interval from 0.5 to 6 hours. This shows that all the amino acid residues present are in their natural (L) configuration, and

it yields conclusive evidence that the molecule contains the residue of aspartic acid, and not that of asparagine

Degradation according to Edman (PTIC) revealed the N terminal sequence H Asp—Arg—Val—Tyr—, the following steps (5, 6, and 7) could not be interpreted unequivocally The C terminal sequence was obtained by the action of carboxypeptidase This enzyme first released leucine then an amino acid that was tentatively identified as histidine, and, last phenylalanine Thereupon the reaction terminated This suggests the C terminal sequence —Pro—Phe—His—Leu OH

Acid hydrolysis confirmed this conclusion Action of concentrated hydrochloric acid at 37°C for 3–4 days yielded 60% of the theoretical amount of the dipeptide H His—Leu OH (sequence obtained by Sanger's DNP method) Another dipeptide, H Arg—Val OH, was also isolated and identified

Action of FDNB on the total tryptic digest of Val^5 angiotensin I yielded DNP aspartic acid and DNP valine Only two peptides could be detected one was identified as H Asp—Arg OH, the other was a peptide with N-terminal valine This confirms the N terminal sequence H Asp—Arg—Val— already deduced from the Edman degradation

Reaction of the total chymotryptic digest with FDNB gave DNP aspartic acid DNP valine and bis DNP histidine Electrophoresis of the chymotryptic digest revealed the presence of three peptides *Peptide No 1* was the tetrapeptide H Asp—Arg—Val—Tyr OH This expected sequence was confirmed by the action of carboxypeptidase showing the C terminal sequence of this peptide to be —Val—Tyr OH *Peptide No 2* contained the amino acids valine, histidine, proline, and phenylalanine Sanger's procedure identified valine as the N terminal amino acid Edman's degradation showed the sequence to be H Val—His—Pro—Phe OH Carboxypeptidase removed only phenylalanine—according to the specificity of this enzyme Partial acid hydrolysis cleaved the tetrapeptide into the two dipeptides H Val—His OH and H Pro—Phe OH *Peptide No 3* was identified as H His—Leu OH

These experiments allow the unequivocal assignment of the sequence H Asp—Arg—Val—Tyr—Val—His—Pro—Phe—His—Leu OH (Fig 9) to the decapeptide Val^5 angiotensin I It may be recalled that the corresponding octapeptide Val^5 angiotensin II, H Asp—Arg—Val—Tyr—Val—His—Pro—Phe OH, had not been isolated

Figure 12 shows the manner of cleavage of Val^5 angiotensin I by various enzymes

6 *Synthetic Work*

a Val^5 Angiotensin I In a short note, Schwyzer *et al* (1956) announced the preparation of active, synthetic decapeptide material This had been

synthesized by way of condensation of the tetrapeptide derivative Z Asp (NH_2)—Arg(NO_2)—Val—Tyr OH with the hexapeptide ester H Val—His—Pro—Phe—His—Leu OCH_3 using the carbodiimide procedure The first crude decapeptide preparations after removal of the carbobenzoxy and nitro groups by hydrogenolysis, followed by mild, alkaline saponification showed varying degrees of activity (between 20 and 60% of that of pure Val[5] angiotensin I) Later experiments showed that the material was a mixture of Val[5] angiotensin I and Val[5] angiotensin I Asp β amide (Fig 10, IIIa,b) containing other, unidentified impurities

It was found (Schwyzer *et al*, 1958a) that the yields and the purity of the material could be greatly improved by altering the sequence of condensation steps and, especially, by the addition of the *N* terminal dipeptide unit last of all This improved synthesis is described in Fig 13 Both the

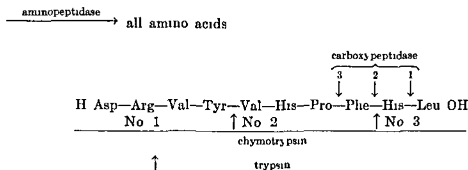


FIG 12 Enzymatic cleavage of Val[5] angiotensin I

decapeptide Asp β amide (Fig 13, *J 1–10,* Fig 10, IIIb) and the decapeptide dicarboxylic acid (Fig 13, *K 1–10,* Fig 10, IIIa) elicit strong pressor activities in the nephrectomized rat (see Section VI, 3), being five and ten times, respectively, more potent than (−) norepinephrine The former compound (Asp β amide) may be converted to the latter (dicarboxylic acid) by mild hydrolysis without producing appreciable amounts of side products, as would be expected to be the case with alkaline hydrolysis of a β ester group Use of the amide group for the protection of the β carboxyl of aspartic acid thus seems to be a very reasonable and practical solution

b Val[5] Angiotensin II This compound (Fig 10, IIa), the natural occurrence of which has not been proved, was first described by Schwyzer *et al* (1957, 1958b) Synthesis followed the pathway outlined in Fig 14 Again, as in the case of the decapeptide, Asp β amides (Fig 14, *H 1–8, I 1–8*) served as intermediates, and were converted to the dicarboxylic acid by mild acid hydrolysis in the last step (→ *J*)

Both compounds are equally potent in the nephrectomized rat (see Section VI, 3) Val[5] angiotensin II Asp β amide was tested in Dr Goldblatt's

laboratories (private communication by Dr S Deodhar) and found to be active in the trained dog test to an extent of 3 2–3 5 Goldblatt units per microgram

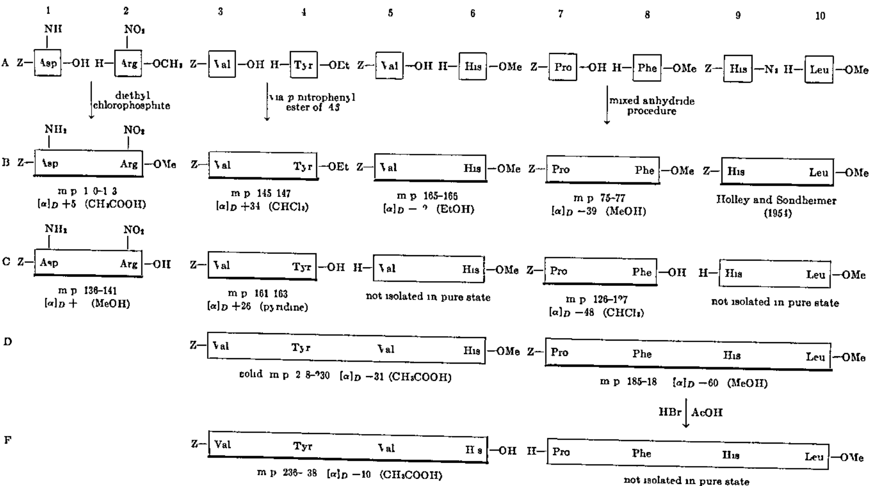
Work with large amounts of synthetic material (several hundred grams) showed that the compounds obtained by freeze drying procedures are not crystalline They retain acetic acid and minute amounts of ammonia (from the buffer used for countercurrent distribution) as well as water Drying in high vacuum over P_2O_5 removes almost all the water (according to microanalysis, Val[5] angiotensin II retains one molecule of acetic acid and two of water, whereas its Asp β amide loses all water), but approximately five molecules of water are regained by both peptides in contact with the atmosphere (cf Fig 14)

This means that the activity of the actual peptide molecule would be somewhat near 1 145 times greater than that of the monoacetate pentahydrate, or about 3 7–4 0 Goldblatt units per microgram (26 Goldblatt units per microgram of nitrogen)

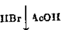
Unpublished experiments show that synthetic Val[5] angiotensin II and its Asp β amide are totally cleaved to the expected peptides by chymotrypsin, trypsin, and carboxypeptidase within short times

The octapeptides were also synthesized by an alternate pathway 2+ (4 + 2), that is a central tetrapeptide was condensed with the *C* terminal dipeptide unit to give a hexapeptide unit, the synthesis being completed by addition of the *N* terminal dipeptide This scheme resembles that used by Schwarz *et al* (1957) (Fig 15) for their synthesis of Ileu[5] angiotensin II, except that we used carbodiimides in the condensation steps Yields seemed to be inferior however, and we reverted to the scheme of condensing dipeptide units in a stepwise manner, starting at the *C* terminal, as being superior [also over the 4 + 4 scheme we had used for the synthesis of Ileu[5] angiotensin II (Fig 16)]

c Ileu[5] Angiotensin II i Work of the Cleveland group The efforts of this group, culminating in the synthesis of Ileu[5] angiotensin II (which seems to be the angiotensin not only of the horse, but also of the hog), are described in a number of papers (Bumpus *et al*, 1957, 1958, Page *et al*, 1957, Schwarz *et al*, 1957) The main points are shown in Fig 15 Four dipeptides were prepared by the method of mixed anhydrides Then a center tetrapeptide derivative, *E 3–6* was prepared, and extended toward the *C* terminus The resulting hexapeptide derivative, *G 3–8*, was then completed at its *N* terminus to the octapeptide derivative *H 1–8* Saponification led to the dicarboxylic acid *I 1–8* Carbobenzoxy and nitro groups were removed by catalytic hydrogenation *J 1–8*, finally, was purified by countercurrent distribution by removing what seem to have been inactive impurities (40% of the total nitrogen) The octapeptide material was not isolated The



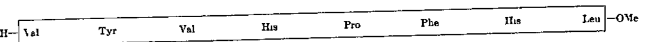
1 2 3 4 5 6 7 8 9 10
A Z—Asp—OH H—Arg—OCH3 Z—Val—OH H—Tyr—OEt Z—Val—OH H—His—OMe Z—Pro—OH H—Phe—OMe Z—His—N3 H—Leu—OMe
diethyl chlorophosphite
via p nitrophenyl ester of 43
mixed anhydride procedure
B Z—Asp Arg—OMe Z—Val Tyr—OEt Z—Val His—OMe Z—Pro Phe—OMe Z—His Leu—OMe
m p 1 0-1 3 [α]D +5 (CH3COOH)
m p 145 147 [α]D +34 (CHCl3)
m p 165-166 [α]D – ? (EtOH)
m p 75-77 [α]D −39 (MeOH)
Holley and Sondheimer (1954)
C Z—Asp Arg—OH Z—Val Tyr—OH H—Val His—OMe Z—Pro Phe—OH H—His Leu—OMe
m p 136-141 [α]D + (MeOH)
m p 161 163 [α]D +26 (pyridine)
not isolated in pure state
m p 126-1?7 [α]D −48 (CHCl3)
not isolated in pure state
D Z—Val Tyr Val His—OMe Z—Pro Phe His Leu—OMe
solid m p 2 8-?30 [α]D −31 (CH3COOH)
m p 185-18 [α]D −60 (MeOH)
HBr AcOH
F Z—Val Tyr Val His—OH H—Pro Phe His Leu—OMe
m p 236- 38 [α]D −10 (CH3COOH)
not isolated in pure state



solid m p 150-1 0 CCD MeOH/H_2O/$CHCl_3$/CCl_4 (15 5 11 9) $K = 0\ 44$ $[\alpha]_D$ −73 (EtOH)

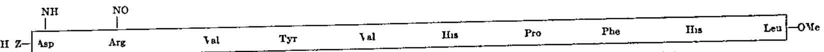
HBr | AcOH

G

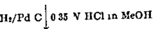


CCD 4 MeOH/H_2O/$CHCl_3$/C_6H_6 (3 27 82 18) $K = 0\ 40$ $[\alpha]_D$ −70 (MeOH)

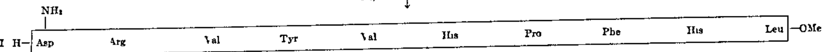
H



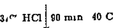
solid m p 180-195 (dec) CCD 4 MeOH/H_2O/$CHCl_3$/CCl_4 (15 5 16 4) $K = 0\ 58$ $[\alpha]_D$ −55 (0 01 N HCl in MeOH)



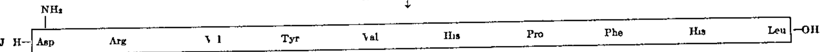
I



di HCl salt not purified



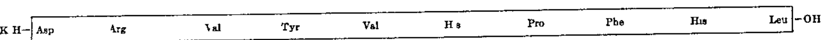
J



solid (CH_3COOH 5H_2O) CCD n BuOH/0 4 M ammonium acetate pH 7 0 $K = 2\ 5$ $[\alpha]_D$ −68 (H_2O)

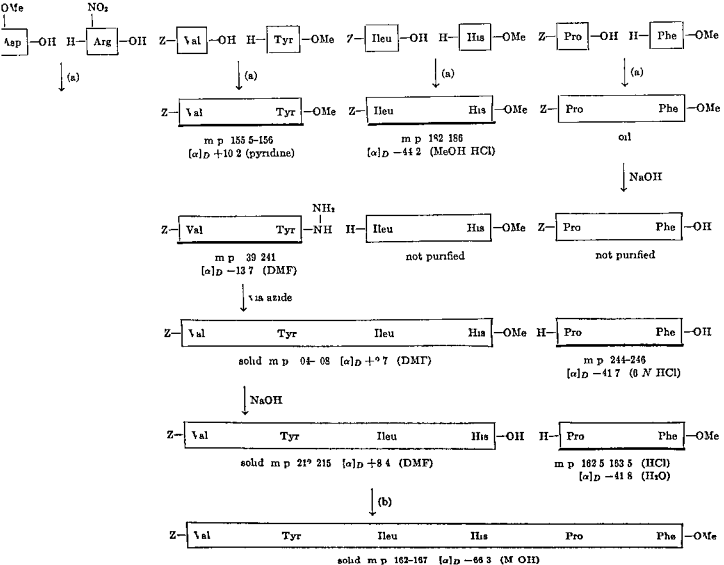
+

K



solid (CH_3COOH 5H_2O) m p > 2 0 (dec) CCD same system K = 0 8 $[\alpha]_D$ −67 5 (H O)

Fig 13 Synthes s of Val[5] ang otensin I (Schw3 z r *et al* 1958a) Unl ss otherwise ndicated condensation was accomplished by the carbod imide procedure and carbobenzoxy groups were removed by cat lytic hyd ogenation E cept in step I → J + K s ponification was effected by alkali See Fig 4 legend


OMe
NO2
Asp—OH H—Arg—OH
Z—Val—OH H—Tyr—OMe
Z—Ileu—OH H—His—OMe
Z—Pro—OH H—Phe—OMe
(a)
Z—Val Tyr—OMe
m p 155 5–156
[α]D +10 2 (pyridine)
Z—Ileu His—OMe
m p 182 186
[α]D −44 2 (MeOH HCl)
Z—Pro Phe—OMe
oil
NaOH
Z—Val Tyr—NH—NH2
m p 39 241
[α]D −13 7 (DMF)
H—Ileu His—OMe
not purified
Z—Pro Phe—OH
not purified
via azide
Z—Val Tyr Ileu His—OMe
solid m p 04– 08 [α]D +°7 (DMF)
H—Pro Phe—OH
m p 244–246
[α]D −41 7 (6 N HCl)
NaOH
Z—Val Tyr Ileu His—OH
solid m p 21° 215 [α]D +8 4 (DMF)
H—Pro Phe—OMe
m p 162 5 163 5 (HCl)
[α]D −41 8 (H2O)
(b)
Z—Val Tyr Ileu His Pro Phe—OMe
solid m p 162–167 [α]D −66 3 (M OH)

F

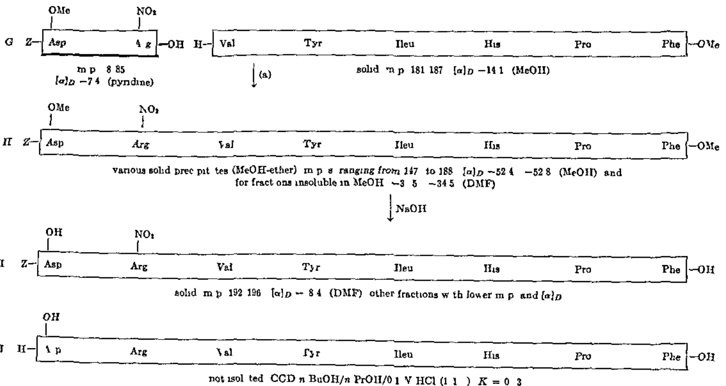
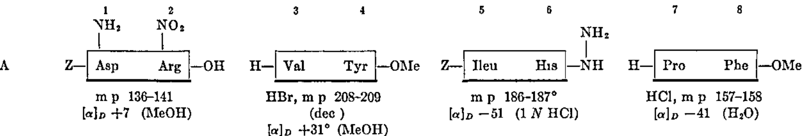
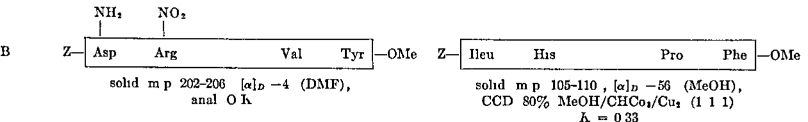
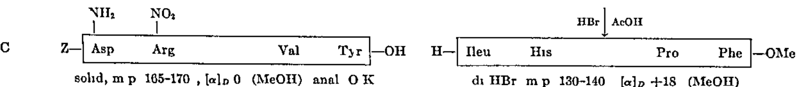


Fig 15 Synthesis of *Ileu⁵ angiotensin II* (Schwarz *et al* 1957) Cleavage of carbobenzoxy and nitro groups by catalytic hydrogenation (a) Condensation by mixed anhydride procedure (EtOCOCl) (b) Condensation by means of diethyl chlorophosphite See Fig 4 legend


A
1 2 3 4 5 6 7 8
NH₂ NO₂
Z— Asp Arg —OH
m p 136–141
[α]D +7 (MeOH)
H— Val Tyr —OMe
HBr, m p 208–209
(dec)
[α]D +31° (MeOH)
NH₂
Z— Ileu His —NH
m p 186–187°
[α]D −51 (1 N HCl)
H— Pro Phe —OMe
HCl, m p 157–158
[α]D −41 (H₂O)


B
NH₂ NO₂
Z— Asp Arg Val Tyr —OMe
solid m p 202–206 [α]D −4 (DMF),
anal O K
Z— Ileu His Pro Phe —OMe
solid m p 105–110 , [α]D −56 (MeOH),
CCD 80% MeOH/CHCo₃/Cu₂ (1 1 1)
K = 0 33
HBr AcOH


C
NH₂ NO₂
Z— Asp Arg Val Tyr —OH
solid, m p 165–170 , [α]D 0 (MeOH) anal O K
H— Ileu His Pro Phe —OMe
di HBr m p 130–140 [α]D +18 (MeOH)

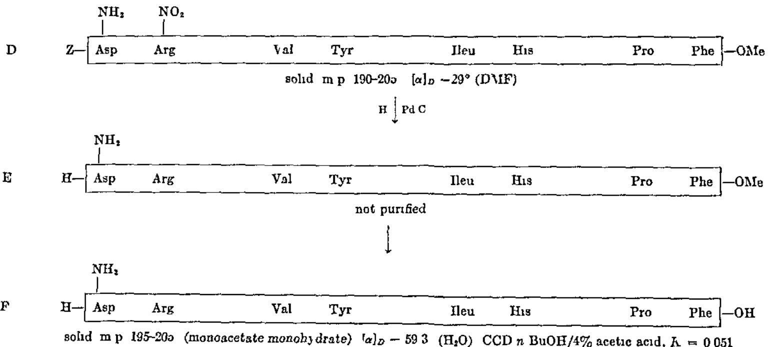


FIG 16 Synthesis of Ileu[5] angiotensin II Asp β amide (Rittel *et al* 1957b Schwyzer *et al* 1957 *Condensations* (except *A 5–6 + A 7–8*) were effected by *the carbodiimide procedure* The most recent physical data are given See Fig 4 legend

activity is reported to be between 50,000 and 55,000 pressor units per milligram of nitrogen (Bumpus *et al*, 1956), 9500 Goldblatt units

The relatively high content of impurities accumulating during the last steps of the synthesis may, in the opinion of the author, be due to two facts First, the mixed anhydride procedure was used to condense the dipeptide *G 1–2* to the hexapeptide *G 3–8* It has been observed (Vaughan and Eichler, 1953) that this procedure may (especially in the presence of traces of water, as in this case) lead to partial racemization (here, of the residue of arginine) Second, alkaline saponification of peptides of aspartic acid β esters often causes formation of cyclic imides (succinimides) which reopen to give mixtures of Asp α and Asp β peptides (Sondheimer and Holley, 1954, Battersby and Robinson, 1955)

Additional interesting information on the preparation of optically pure intermediates is to be found in the paper of Schwarz and Bumpus (1959)

ii Work of the Basle group A synthesis of the Asp β amide of Ileu[5] angiotensin II (Fig 10, Ib) was described in two papers by Rittel *et al* (1957a, b) It followed the path outlined in Fig 16

More recent work (Schwyzer *et al*, 1957) using acid hydrolysis of the amide *E 1–8* as in the case of the Val[5] compounds (Section IV, 6, a, b), led to Ileu[5] angiotensin II (Fig 10, Ia), plus Ileu[5] angiotensin II Asp β amide (Fig 10, Ib) Owing to the starting material, both the compounds contain 7% of *allo* isoleucine residues [determined by Brenner and Weber, (1959), using the technique of Moore *et al* (1958)] The physical constants are *Ileu[5] angiotensin II* $[\alpha]_D^{23}$ $-67\ 3° \pm 2$ (c 1 13, 1 N acetic acid), m p 210–250° (dec), analyses as trihydrate, CCD n BuOH/0 4 M ammonium acetate, pH = 7 2, K = 0 265 *Ileu[5] angiotensin II Asp β amide* $[\alpha]_D^{27}$ $-58\ 6° \pm 1°$ (c 1 06, 1 N acetic acid), m p 195–205° (dec), analyses as monoacetate monohydrate, CCD in same mixture, K = 0 710

In view of the related work of the Cleveland group, these results (obtained by Dr W Rittel) have hitherto not been published They are listed here as the sole source available at present of this kind of physical and chemical information on Ileu[5] angiotensin II

V Synthetic Analogs of Angiotensin

The preparation of polypeptides analogous to the angiotensins or to "polypeptide renin substrate" might serve a number of purposes Of these, the most conspicuous are (1) the correlation of biological activity to structure, (2) the possible discovery of compounds of greater potency or longer action (3) the possible discovery of antagonists toward angiotensin (blocking of receptor sites), (4) the possible discovery of compounds that inhibit renin If they could be realized, points (3) and (4) would be of great value in combating certain types of hypertensive diseases In spite of the con

siderable number of analogous peptides already prepared, information on point (1) only has been obtained until now

No details on the preparation of analogs of angiotensin other than the Asp β amides (Section IV, 6, a, b, and c, II) have hitherto appeared, although a number of the compounds prepared by the Swiss group have been mentioned or listed (Schwyzer *et al*, 1957, Schwyzer, 1958, 1959) We hope to be able to publish the work *in extenso* in the near future in *Helvetica Chimica Acta* The sections that follow (V, 1–3) give a brief account of the compounds prepared, including their biological activity in the nephrectomized rat (as assayed by Drs Gross and Turrian of CIBA Ltd, Basle cf Gross and Turrian, 1960) The differences in biological activity encountered were mainly quantitative, not qualitative The fact that no antagonists of angiotensin were found among the two dozen synthetic analogs deserves special mention

1 Functional Derivatives of Angiotensin

The synthetic compounds with altered functional groups are listed in Fig 17 Compounds II and III clearly demonstrate that exchange of aspartic acid for asparagine in position 1 of the peptide chain leaves the activity unaltered, within experimental error This unexpected phenomenon seems to be quite general, as a similar behavior is shown by the corresponding compounds II VI, and VII (Fig 18), and I and II (Fig 19)

Greater specificity seems to be connected with the C terminal carboxyl group, for an alteration at this point leads to a strong reduction of activity esterification by tenfold, and formation of the amide even by thirty times (compounds IV, V, Fig 17)

Substitution of the guanidino function of the residue of arginine by the nitro group (with subsequent reduction of the basicity at this point) as in compound VI (Fig 17), lead to a slight diminution of the activity Sterical factors seem to play an important role at this point as demonstrated by the results of the replacement of arginine by ornithine and lysine (compounds II V, Fig 18)

2 Analogs of Angiotensin II Containing Other Amino Acid Residues

The fact that amide formation with the β carboxyl of Asp[1] has no influence on activity might lead to the conclusion that position 1 of the peptide chain as such is not connected with any great specificity This is at least partly true, because the replacement of Asp[1] by Gly (compound I, Fig 18) reduces activity only by a factor of 2

The second position seems to be more specific One might tentatively conclude from compound VI (Fig 17) and from compounds II and V (Fig 18) that an amino group on the δ (but not ε) carbon of the second amino

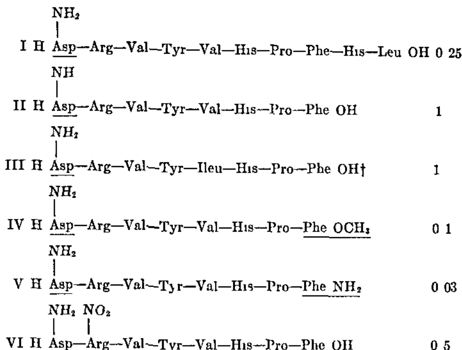


FIG 17 Functional derivatives of angiotensin Structural changes are indicated by underlining The figures at the right denote the pressure response of the nephrectomized rat (Gross and Turrian 1960) relative to synthetic Val[5] angiotensin II and to the equally active Ileu[5] angiotensin II taken as 1 (about twenty times norepinephrine) † Contains 7 % *allo* isoleucine residues

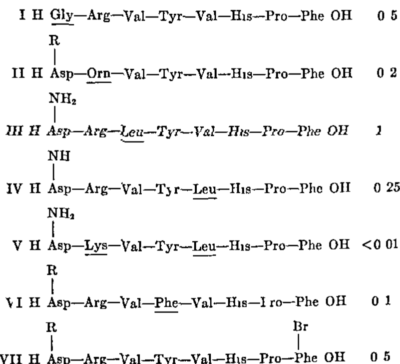


FIG 18 Analogs of angiotensin II containing other amino acid residues (cf Fig 17 legend) —R = —OH and —NH2 —Phe(Br)— = L p bromophenylalanine

acid is essential for strong activity, although this has to be borne out by more examples in the future

The two amino acid residues with aliphatic side chains in positions 3 and 5 of the peptide (Val^3 and Val^5 or $Ileu^5$) differ somewhat in their specificity If the branching is moved outward from the β to the γ carbon atom (replacement of valine or isoleucine by leucine), activity remains undiminished when this is done in position 3, but is reduced to one fourth by a corresponding change in position 5 (compounds III and IV, Fig 18)

A great fall of activity by replacement of Arg by Lys^2 has been attributed mainly to this interchange, although compound V (Fig 18) contains not only the residue of lysine in position 2, but also that of leucine in position 5, so that a small part of the drop in activity must be related to this latter feature Even then, the influence of the Arg Lys interchange is very impressive, contrasting strongly with the behavior of the corresponding Arg and Lys vasopressins which are equally active

Position 4 also displays a strong degree of specificity the p OH group in Tyr^4 is essential for full pressor action A tenfold reduction of activity is produced by replacement of Tyr^4 by Phe^4 (compound VI, Fig 18) This behavior parallels that of the corresponding analogs in the oxytocin and vasopressin series (Taquenoud and Boissonnas, 1959, Bodanszky and du Vigneaud, 1959)

Although substitution of the C terminal carboxyl group (by the rather stable carboxamide group) produced quite strong effects, substitution of the p hydrogen of the C terminal phenylalanine by bromine (introduction of L p bromophenylalanine) brings about only a small change in activity (compound VII, Fig 18) This is comparable to that of esterifying the C-terminal carboxyl group (maybe the ester grouping is rather rapidly cleaved, producing the fully active octapeptide *in vivo*)

3 Peptide Chain Homologs Related to the Angiotensins

The expression "peptide chain homologs" is here used—in a manner similar to 'polymer homologs" in polymer chemistry—to designate peptides differing from one another by the number of amino acid residues comprising the chain Peptide chain homologs of angiotensin, then, are peptides comprising sequential features of angiotensin I or II, but varying in the length of the chain

Compound I (Fig 19) represents homologs of Val^5 angiotensin II and its Asp β amide, they differ by two amino acids, the C terminal dipeptide sequence —Pro—Phe— occurring twice The product retains almost no pressor activity this demonstrates (1) that the C terminal —Phe OH must be at a certain distance from other essential parts of the molecule (Arg ?), and (2) the great specificity of the converting enzyme, because

this decapeptide, unlike angiotensin I or its Asp β amide, is not converted to an active entity in this particular *in vivo* assay

Compound II (Fig 19) represents nonapeptides containing a second residue of tyrosine next to the original one These compounds also show a very low activity (explainable by the elongation of the distance Arg to Phe?) Unlike a comparable compound in the oxytocin field (Guttmann *et al*, 1957), *this shows no signs of antagonism*

The previously demonstrated fact (Section V, 2) that position 1 is not responsible for the activity holds also *in the case of compound III* (Fig 19), where the first amino acid is absent This heptapeptide shows half the original activity The same compound, in the Ileu^5 series, had been pre

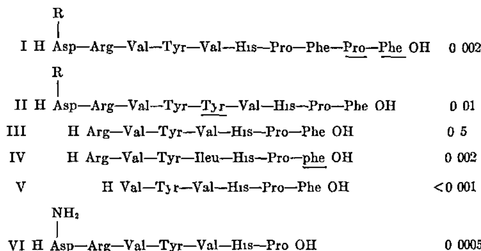


FIG 19 Peptide chain homologs related to angiotensin (cf Fig 17 legend) —R = —NH_2 and —OH, Phe = L isomer, phe = D isomer

pared from natural octapeptide by Skeggs and Kahn (1958) and had been shown to be active in a very similar range

Further reduction of the length of the chain from the *N* terminal (omission of Arg) leads to the fully inactive hexapeptide (compound V, Fig 19) (cf Skeggs and Kahn, 1958) Equal inactivation results from the cleavage of —Phe OH from the *C* terminal as Lentz *et al* (1956) have demonstrated in the case of Ileu^5 angiotensin II (degradation with carboxypeptidase) This phenomenon has been demonstrated to hold also in the Val^5 series (compound VI, Fig 19)

The only demonstration *of an effect of the optical configuration* on activity has been carried out on the heptapeptide (compound IV, Fig 19), where an exchange of the L form of phenylalanine for the D form was dramatic enough complete inactivation followed Residual activity may, of course, be due to fractions of 1% of L phenylalanine contained in the starting material

VI Pharmacology of Angiotensin

By H TURRIAN

1 Methods of Assay

Although the existence of synthetic preparations of hypertensin now allow a gravimetric determination to be carried out, biological methods are still in use in particular for the demonstration of natural angiotensin These methods may be classed in two groups (1) Stimulation of smooth muscle in *the isolated organ* (2) Measurement of arterial blood pressure in *the whole animal*

a Assay on the Isolated Organ Various methods using isolated organs (vascular preparation of the toad, guinea pig ileum, and rat uterus) have been described for the quantitative evaluation of angiotensin (Fasciolo and Taquini, 1947, Picarelli *et al*, 1954, Prado *et al*, 1954, Schwarz and Page, 1954) The wide variations in sensitivity encountered in the course of an assay (Gross and Turrian, 1960), as well as the lack of specificity of these methods, largely counterbalance the slight advantage they may possess in simplicity and rapidity of preparation, as compared with experiments on the intact animal

b Assay in the Whole Animal Measurement of the hypertensive effect of angiotensin in the anesthetized animal is a convenient method of determination The rat cat, or dog is a suitable experimental animal which can be pretreated by various operations or drugs in order to increase its sensitivity to angiotensin, e g section of the "buffer nerves" (MacCubbin and Page 1952), nephrectomy (MacCubbin and Page, 1954, Gross and Lichtlen, 1958), destruction of the central nervous system (Friedmann *et al*, 1938) using sympathicolytics (Dekanski, 1954) or ganglion blockers (Peart, 1955)

The rat is the most suitable laboratory animal for the quantitative evaluation of this activity Gross and Lichtlen, (1958) have shown that, as the curves obtained using the nephrectomized rat are more regular and the results more consistent rats subjected to nephrectomy seem to be particularly indicated for angiotensin bioassay

2 Experiments in Vitro

Ludueña demonstrated as early as 1940 that angiotensin caused contraction of practically all smooth muscle organs, although with considerable variations in sensitivity depending on the organ examined, it is also well known that its action does not parallel that of either the adrenergic or cholinergic substances, and that it may be classed with the musculotropic drugs (Braun Menendez, 1956)

Angiotensin II produces pronounced vasoconstriction in concentrations over 10^{-9} gm /ml in the vessels of isolated rabbit hind legs Similar concen

trations provoke coronary vasoconstriction and a weak positive inotropic and chronotropic effect on the isolated heart of the cat or rabbit (Langendorff method, Meier *et al*, 1957, 1958, Kuschinsky and Lullmann, 1959) This coronary effect can be antagonized by such vasodilators as adenosine, papaverine, chlorpromazine, and Apresoline It will shortly be seen that this coronary effect is not found with the intact animal

In the isolated heart of the cat and rabbit, angiotensin I is somewhat less active than the octapeptide, in other isolated organs, especially the guinea pig ileum and rat uterus, the difference is even more marked and the decapeptide has an oxytocic action ten to twenty times weaker than that of angiotensin II (Carlini *et al*, 1958, Gross and Turrian, 1960) As Skeggs (1954a, 1956a), Helmer (1956), and Bumpus (1956) have shown, this is partly due to the fact that angiotensin I has to be transformed into angiotensin II before it becomes active The work of Carlini *et al* (1958), however, would suggest that this is not the only factor involved and that the deca and octapeptides may have different properties, depending on the organ under examination The fact that the presence of calcium ions is necessary for the oxytocic action of angiotensin II to develop (Renson *et al*, 1959) also indicates that factors other than the transformation of the deca to the octapeptide under the influence of the "converting enzyme" may well play some part in the problem

3 Hypertensive Action

The specific effect of angiotensin, i e a rise in blood pressure of brief duration, may be demonstrated in various species of animal as well as in man Doses of 0 01 μg /kg or more of Val^5 angiotensin produce in the rat a rise in blood pressure attaining a maximum within 20-30 seconds and disappearing within 2-5 minutes, the blood pressure elevation increases linearly with dosage up to 10 μg /kg i v , in this range there is no correspondingly marked prolongation of the effect (Gross and Lichtlen, 1958, Gross and Turrian, 1960) The sharp rise in blood pressure produced by angiotensin closely resembles that caused by norepinephrine, here too, there is no secondary fall in pressure

In anesthetized animals a slight diminution in the heart rate is normally observed in response to isolated doses of angiotensin, during continuous injections, slight bradycardia is associated with the rise in pressure, whereas during infusions mild tachycardia may occur (Page *et al*, 1957) In human subjects, bradycardia, probably of reflex origin, is observed in response both to isolated doses and to continuous infusion (Bock *et al*, 1958a, Finnerty *et al*, 1959, Lichtlen *et al*, 1959) The cardiac output does not change (Lichtlen *et al*, 1959) or is only slightly diminished (Gersmeyer *et al*, 1958)

As regards their hypertensive action, Val^5 hypertensin II and its amide

are about twenty times more active than norepinephrine in the intact and in the nephrectomized rat Val[5] hypertensin I has half the activity of the octapeptide, and the corresponding amide is about four times less potent In man, Val[5] hypertensin II is also more active (five to ten times) than norepinephrine, and in particular its action is of longer duration (Bock *et al*, 1958a, Finnerty *et al*, 1959) As is the case with other polypeptides (Woolley and Merrifield, 1958), rather important structural changes in the angiotensin molecule may be made without much alteration in the hypertensive action, which remains qualitatively of the same type (Gross and Turrian, 1960) (Section V)

a Tachyphylaxis In both animals and man, repeated administration of a dose of angiotensin insufficient to cause a maximal effect leads to a hypertensive response, the intensity of which does not diminish even if there is only a short interval between the successive doses (4 or 5 minutes) Thus, no tachyphylaxis occurs (Bumpus *et al*, 1957, Meier *et al*, 1957) This could perhaps be because angiotensin has only a very short duration of action (Gross, 1958) The hypertensive effect of an additional dose of hypertensin administered during continuous infusion of hypertensin is indeed markedly diminished Similarly, the effect of hypertensin is greatly diminished or even abolished after administration of high doses of renin, whereas that of epinephrine is not (Goldblatt *et al*, 1953, Page *et al*, 1957)

b Continuous Injection Continuous intravenous injection of angiotensin causes a rise of blood pressure of the same duration as the infusion (Braun Menendez *et al*, 1940) The increase in blood pressure and the return to normal take place rapidly (Page *et al*, 1957, Meier *et al*, 1957) During the infusion, the blood pressure remains at a high level in a more consistent manner than when norepinephrine is infused In animals and man, continuous infusion leads to a maximal response of about 30–40 mm Hg, which cannot be augmented by increasing the dose Page *et al* (1957) have shown that nervous compensatory mechanisms are active in opposing the further blood pressure elevation since administration of a ganglion blocker during an infusion of hypertensin leading to a maximal response produces a rise in blood pressure Angiotensin, however, has no effect on either the baroreceptors or the chemoreceptors (MacCubbin *et al* 1957, Bianchi *et al*, 1959)

c Modifications in the Response to Angiotensin It is well known that sympathicolytics do not modify the hypertensive action of angiotensin whereas ganglion blockers reinforce it, as they do for adrenergic substances Recently, Meier *et al* (1957) have shown that hypotensive agents such as chlorpromazine and reserpine do not diminish the hypertensive action of angiotensin, hydralazine is also without effect (Page *et al*, 1957, Gross and Turrian 1960), although this compound diminishes the vascular response to vasoconstrictor substances

Many attempts have been made to demonstrate a change in the sensitivity to angiotensin during chronic hypertension in both animals and man In rats pretreated for 2 or 3 weeks with high doses of cortexone or aldosterone together with sodium chloride, the action of hypertensin is potentiated (Gross and Lichtlen, 1958, Gross, 1958) It is not necessary, however, that animals develop hypertension (as does the rat under these experimental conditions) in order to observe this potentiation, rabbits, for example, do not react to overdosage of cortexone by developing hypertension, and yet, here again, the animal's sensitivity to hypertensin is potentiated The effect of adrenergic substances is likewise enhanced, and what obviously occurs is an increase in sensitivity to all hypertensive stimuli it is possible that a rise in the sodium, potassium, and water content of the arterial walls is partly responsible for this (Gross and Lichtlen, 1958, Gross and Schmidt, 1958) In this context, it should be remembered that diuretics such as theophylline ethylenediamine and chlorothiazide antagonize the action of hypertensin (Bianchi *et al*, 1959)

In animals with experimental hypertension of renal origin, the hypertensive action of hypertensin can also be potentiated, but results are not consistent (Verney and Vogt, 1938, Ogden *et al*, 1940, Page, 1941) Contradictory results have similarly been obtained in patients with chronic hypertension Whereas Battro *et al* (1941) found no differences between normotensive and hypertensive subjects as regards their sensitivity to angiotensin, Imhof *et al* (1959) report increased sensitivity in the case of hypertensives At the same time, in hypertensives the response to epinephrine is also enhanced (Greisman, 1956, Duff, 1957)

4 Other Circulatory Actions

a Coronary Artery It has been mentioned that angiotensin produces a diminution of the coronary flow in the isolated rabbit and cat heart, on the other hand, vasodilation is observed in the anesthetized animal (cat and dog) (Gross and Turrian, 1960, Potgieter *et al*, 1959), though this is less marked than with norepinephrine Sometimes slight secondary vasoconstriction is found, but this is never so pronounced as with vasopressin In man electrocardiographic examination has shown no changes attributable to hypoxemia (Finnerty 1959, Lichtlen *et al*, 1959)

b Peripheral Blood Flow Although Corcoran and Page (1940) found no change in cutaneous temperature in response to angiotensin infusions in the dog, Bock *et al* (1958a) have reported a definite diminution in the peripheral blood flow in man (as determined by measurement of temperature), the muscular blood flow exhibiting a slight increase concomitant with the rise in blood pressure

Imhof *et al* (1959) have also referred to a lowering of the cutaneous

temperature in man under the influence of angiotensin The same authors were unable to distinguish any change in the capillaries of the eye fundus in response to angiotensin, but these vessels, like those of the brain, are not very sensitive to the influence of drugs

c Pulmonary Blood Pressure Carlier *et al* (1958), Maxwell *et al* (1959), and Reid and Jenkins (1948) observed no rise in the pulmonary blood pressure after hypertensive doses of angiotensin in the dog On the other hand, in other animal species (cat, rabbit) and in man, a rise in pulmonary blood pressure is found which is relatively as great as that occurring in the systemic circulation (Reid and Jenkins, 1948, Gersmeyer *et al*, 1958, Lichtlen *et al*, 1959)

d Renal Circulation Whether the activity of angiotensin on renal circulation is examined by methods of direct perfusion Barac (1959), using the thermostromuhr (Gross and Turrian, 1960) or a flowmeter (Barer, 1959), or by clearance methods (Corcoran and Page 1940, Bock *et al*, 1958b), a diminution of renal blood flow is observed Whereas Corcoran and Page (1940) attribute these modifications to vasoconstriction of the vasa efferentia, Bock and Krecke (1958) consider that they are primarily due to an action exerted on the preglomerular vessels Angiographic examinations carried out by Daniel *et al* (1954) have shown that at least in the rabbit and under the influence of renin preglomerular vasoconstriction may well occur

5 Action on Renal Function

The effect of angiotensin on urinary output varies according to the species of animal, its state of hydration, and the dose administered, both diuretic and antidiuretic activity have been ascribed to this polypeptide (for detailed bibliography see Gross, 1958) Much recent work, however, seems to indicate that the amides of Ileu[5] or Val[5] angiotensin II (Barac, 1958, 1959, Bock *et al* 1958b, Gross and Turrian, 1960) must be regarded as producing an antidiuretic effect if administered by infusion in doses adequate to provoke hypertension In animals, sodium and potassium excretion decreases at the beginning of the infusion period, but a marked secondary increase in sodium elimination is observed (Uranga 1956, Gross and Turrian 1960) Bock *et al* (1958b) have likewise reported antidiuresis with elevation of the urinary potassium concentration the sodium concentration remaining, largely unchanged however

It appears possible that angiotensin, like vasopressin exerts an antidiuretic effect this effect might be attributed, as in the case of epinephrine and norepinephrine (Pickford and Watt, 1951), to renal vasoconstriction leading to reduction in the glomerular filtration rate This hypothesis does not, however, take into account the increase in sodium excretion noted in

animals, and it would be interesting to find out whether the antidiuretic and hypertensive effects of angiotensin are not independent

6 General Remarks and Conclusion

Although angiotensin exhibits well established effects on the circulation, its precise physiological significance is far from clear The fact that *in vitro* renin liberates hypertensin from an α_2 globulin fraction of the plasma does not prove that the same occurs *in vivo*, and still less does it prove that angiotensin plays any part in the regulation of the blood pressure or that it is involved in the pathogenesis of hypertension Gross (1958) has suggested that the renin hypertensin system plays a part in the regulation of renal function and that connections exist between this system and the active substances produced by the adrenals This hypothesis still requires a great deal of experimental confirmation, and in particular the role played by angiotensin itself in these functions remains to be elucidated

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Symposium on Vitamin A and Metabolism

in honor of Professor P Karrer

A collection of invited papers presented at Burgenstock Switzerland on May 23 24 and 25 1960 This symposium was sponsored by F Hoffmann La Roche Basle

SYMPOSIUM ON VITAMIN A AND METABOLISM

Bürgenstock May 23-25, 1960

Opening Remarks

P KARRER

The research department of Hoffmann La Roche, and its representative Dr O Isler, had the happy idea of arranging this symposium, at which experiences concerning the biological behavior and the physiological effects of vitamin A might be exchanged The fact that this is being held twenty nine years after the elucidation of the constitution of vitamin A, and even fourteen years after its synthesis, shows that the biological problems connected with this vitamin are obviously of a rather complicated kind

I am sorry to say that several of the pioneers of vitamin A investigation are no longer among us I remember especially Sir Ian Heilbron, who a long time ago took part in the chemical investigations on vitamin A and also Professor I M Drummond, who was among the first to study the biological behavior of this vitamin in animals We are also missing here Professor Sir Edward Mellanby who died five years ago, he made very important studies on vitamin A deficiency and its influence on bones, nerves, and other organs I am very glad that I can welcome here Lady Mellanby, who has kindly accepted an invitation to our symposium

It is a special pleasure to me that another colleague, Professor H von Euler, who participated in the first investigations of vitamin A in a decisive way, can today be among us As early as thirty years ago he conducted biological examinations of vitamin A preparations which were preliminary to the isolation and purification of this compound I owe him personally a debt of gratitude

May I be permitted to mention also Dr Thomas Moore as one of those colleagues who participated in vitamin A investigations at a very early time He made important observations about its biological behavior, and he is also the author of an excellent book on vitamin A We are glad that he is with us today

Also among pioneers of the vitamin A investigations is Dr R A Morton who has published many interesting papers relating to the absorption spectra and the physical and chemical properties of vitamin A preparations

May I finally welcome Professor George Wald, who discovered vitamin A in the eye and who afterward made the classic investigations that cleared up to a considerable extent the functions and the importance of vitamin A in the visual processes We in Zurich are glad, and even a little proud of the fact, that it was in our laboratory that Dr Wald made his first extractions of vitamin A from the eye and proved its occurrence in these organs

If I mentioned the special merits of some colleagues who took part in the vitamin A investigation from its beginning, I no less acknowledge the work of others who began it at a somewhat later time All the colleagues invited to this symposium have enriched our knowledge of the vitamin A group and I welcome here J G Bieri, N D Embree, J Ganguly, U Gloor, J Glover, R Grangaud, R S Harris, P L Harris, B C Johnson, O Isler, M Kofler, S H Rubin, J Tiews, H Wilkinson, O Wiss, G Wolf, and J Wursch

Vitamin A was, as you know, the first vitamin to have its constitution clarified For this reason it possesses a special position in vitamin research But it was also the first vitamin to have its provitamins, the α, β- and γ carotenes, completely elucidated with regard to their structure Thus, for the first time the connection between the structure of a vitamin and its provitamins could be expressed by chemical formulas

Scientific problems and their general importance can be understood only if we recall the mentality of the era in which they arose and in which they were solved Thus, the rapid progress made in the chemistry of vitamins in the 1930's caused at that time a sensation, which the generation of today can hardly imagine It remained for those investigations to eliminate completely the widespread hypothesis that vitamins are special forms of energy and to prove that they are chemical compounds of characteristic structure

Important progress was made in the chemistry of vitamin A when it was discovered that these polyenes can occur in *cis trans* isomeric forms and that there are among the *cis* compounds isomers which are sterically hindered and therefore labile, whereas other *cis* compounds may be stable like the all *trans* forms These isomers also exhibit varied biological behavior It will certainly be a long time before the physiological and biological problems connected with these facts are completely cleared up

We are fairly well informed about the physiological functions of the water soluble vitamins because they are parts of enzymes, the reactions of which are pretty well known On the other hand, we have very little information about the biological role of the fat soluble vitamins This is true also for vitamin A For this reason it should be very useful that these problems are now to be discussed by experienced specialists Our program covers a number of specific subjects

Synthesis of vitamin A compounds

Physicochemical assay and bioassay of vitamin A compounds
Conversion of provitamins to vitamin A
Absorption, transport, and storage of vitamin A
Degradative metabolism of vitamin A and metabolic functions of vitamin A acid
Function of vitamin A in carbohydrate, mucoprotein, and lipid metabolism
Deficiency symptoms of vitamin A
Vitamin A and vision

It is not my intention to anticipate these lectures Let me only say that as yet we know very little about the functions of vitamin A in the animal body, with the exception of its activity in the visual process It is true that some scientists suppose that vitamin A plays a role in the biosynthesis of cholesterol, squalene, and ubiquinone, but these questions are rather complex, and today it is difficult to answer them completely Neither do we know very much about the formation of vitamin A from its provitamins, although these processes are of more than scientific interest It seems to me that it should not be too difficult to solve this problem experimentally

May I therefore express the hope that the lectures and the discussions of this symposium will help to clear up some problems concerning vitamin A But I also hope that the personal contacts among the colleagues here present may intensify their friendly relations This would certainly be of great benefit for science

I am convinced that I speak in the name of all of us in expressing our most sincere thanks to Hoffmann La Roche, Basle, for having arranged this meeting

The Synthesis and Labeling of Vitamin A and Related Compounds

OTTO ISLER, RUDOLF RÜEGG, ULRICH SCHWIETER, AND JOSEF WÜRSCH

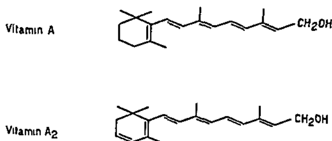
Research Laboratories F Hoffmann La Roche & Co Ltd Basle Switzerland

I Introduction

Ever since the elucidation of the structure of vitamin A by Karrer (Karrer *et al*, 1931) the synthesis of this rather unstable pentaene alcohol has been a challenge to chemists Owing to patient and continuous efforts in many laboratories there seems to be today an excellent picture of the possible synthetic routes to vitamin A This research on the synthesis and biochemistry of vitamin A and its stereoisomers has been the subject of an excellent monograph by Moore (1957) and of a number of reviews (Jones, 1941, Sobotka and Bloch 1944 Milas 1947, Isler, 1950, Baxter 1952, Heilbron and Weedon 1958)

Although we have a wealth of information on its synthesis, relatively little is known about the functions of vitamin A in the body Knowledge on this subject broadened beginning with the advent of radioactive tracer methods It therefore seems justified to take stock of the synthetic methods available, and to examine them as to their usefulness for the preparation of selectively labeled vitamin A compounds These compounds will undoubtedly help the biochemists in their patient and often laborious inves

tigations of the biological pathways that vitamin A and certain related compounds seem to control



Vitamin A is found in many tissues, animal fats, and fish oils Before the synthetic material was available, fish liver oils—where it is often accom

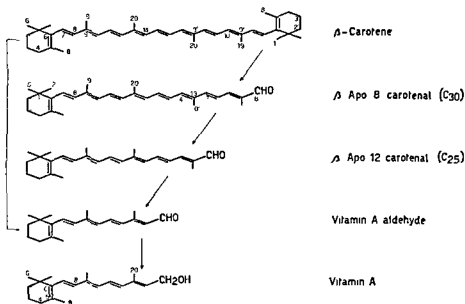


FIG 1 Conversion of β carotene to vitamin A in the animal body

panied by dehydrovitamin A, also called vitamin A_2—were the main source of this vitamin Its deficiency symptoms are associated with night blind ness, xerophthalmia, and the arrest of growth The growth promoting effect of vitamin A is the basis for a most reliable assay method By definition all *trans* vitamin A has a 100% activity, all other compounds investigated have shown less activity than this

The growth promoting activity is not restricted to vitamin A and its isomers, it is also found with certain carotenoids The conversion of β carotene to vitamin A in the animal body has attracted a great deal of atten tion The results of this research are indicated in Fig 1

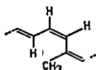
It has been well established (Hunter, 1946, Mattson *et al*, 1947, Wiese

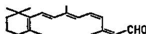
et al, 1947, Glover *et al*, 1948) that this conversion occurs in the intestinal wall via vitamin A aldehyde or retinene, as it is often called Oxidative fission of the β carotene molecule at the central (15,15′) double bond would give rise to two molecules of vitamin A Most of the investigations, however, showed a 1 1 ratio, and only Koehn (1948) and Burns *et al* (1951) have claimed a conversion to two molecules of vitamin A in the presence of α tocopherol Glover and Redfearn (1954) therefore suggested another mechanism, whereby the β carotene molecule is attacked at a terminal double bond, giving rise to the β apocarotenals Successive β oxidation would then result in the formation of one molecule of vitamin A aldehyde from one molecule of β carotene

As indicated in Fig 1 we shall throughout this paper use the same numbering system for vitamin A as was proposed by Karrer and recommended by the International Union of Pure and Applied Chemistry (1947) for the carotenoids

II Vitamin A Stereoisomers

Owing to *cis trans* isomerism at the double bonds, stereoisomers of vitamin A can be formed These isomers can be divided into two categories, the "unhindered *cis*" and "hindered *cis*" types The latter type is often referred to as "Pauling hindrance" Pauling (1939) pointed out that *cis* isomers of the configuration indicated will be very unstable and will tend to assume the *trans* configuration because of severe steric hindrance between the methyl group at C 1 and the hydrogen atom at C-4 For some





11-cis Vitamin A aldehyde
(neo-b-retinene)

time this postulate was so interpreted that compounds with Pauling hindrance were considered to be incapable of existence In the meantime, however, such compounds have been prepared One example is the 11 *cis* vitamin A with hindrance between the C 20 methyl group and the hydrogen atom at C-10 This isomer easily assumes the *trans* form under the influence

of light or iodine, whereas 9 *cis* vitamin A with no such hindrance is rather stable under these conditions

Figure 2 summarizes the vitamin A compounds that have been prepared so far

It was already mentioned that all *trans* vitamin A and its aldehyde are found in many tissues and exhibit the highest biological potency Van Dorp and Arens (1946a) claimed that all *trans* vitamin A acid has the same activity as the alcohol in the rat growth test when administered as its sodium

	X CH$_2$OH mp	X CHO mp	X COOH mp
all trans	62 64	61-62	179 180
13 cis (neo a)	58 60	77	175 176
11 cis (neo b)	oil	63 5 64 4	—
9 cis (iso a)	82 83	64	189 191
11 13 di cis (neo c)	oil	oil	—
9 13 di cis (iso b)	58 59	49 and 85	135 136

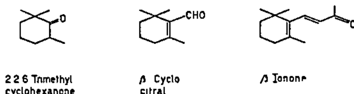
FIG 2 Stereoisomers of vitamin A compounds Asterisk indicates an isomer with Pauling hindrance

salt in a buffered solution The importance of 11 *cis* vitamin A aldehyde in the visual process was shown in a series of thorough and excellent investigations by the schools of Wald and Morton Morton (1957) later reviewed these findings

Although some of the other isomers have been isolated from natural material, there is still some controversy as to whether they are artifacts or indeed occur naturally

III SYNTHESES OF VITAMIN A

Three obvious starting materials suggest themselves for the synthesis of vitamin A, namely, 2,2,6 trimethylcyclohexanone, β cyclocitral, and β ionone



All of these have been used for the preparation of vitamin A For the synthesis of labeled vitamin A with a high specific activity the following requirements will have to be met (1) possible introduction of a label into various positions of the ring system, (2) possible synthesis of vitamin A isomers with an additional label at various carbon atoms of the side chain, (3) good yields

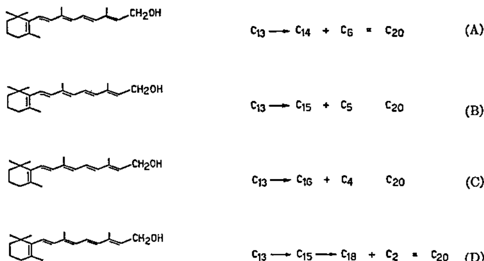


FIG 3 Building principles of vitamin A from β ionone (A) cf Fig 5 (B) cf Figs 6 and 7 (C) cf Fig 8 (D) cf Fig 9

All these requirements are fulfilled with β ionone, which can be obtained by total synthesis from acetone Figure 3 summarizes the effective ways for adding the remaining seven carbon atoms of the vitamin A skeleton

1 *Total Synthesis of β Ionone*

The synthesis of β ionone proceeds via citral, which can be obtained from lemon grass oil or synthesized from acetone

The total synthesis of β ionone via citral offers a wide choice for labeling The procedure was developed in the laboratories of F Hoffmann La Roche & Co in Nutley, New Jersey, and in Basle by Kimel *et al* (1957) and Saucy *et al* (1959) (Fig 4) Acetone is condensed with acetylene in liquid ammonia, the product is hydrogenated and reacted with either diketene or acetoacetic ester For the labeling of C-4, C 5 and C-18 of the vitamin

A skeleton, the acetoacetic ester condensation is to be preferred Primary *methylbutenyl bromide is used in this reaction*, yielding 2 methyl 2 hepten 6-one from which dehydrolinalool is prepared by condensation with acetylene Acylation and rearrangement of the acetate in the presence of silver salts gives citral, which undergoes condensation with acetone in an alkaline medium to form ψ ionone (Tiemann and Kruger, 1893), subsequent cyclization under controlled acidic conditions yielding β ionone (Royals, 1946) By this procedure, C^{14} β ionone with labels corresponding to C 6 and C 7 of vitamin A *was prepared in the Basle laboratories (activity 30 μc/mg)*

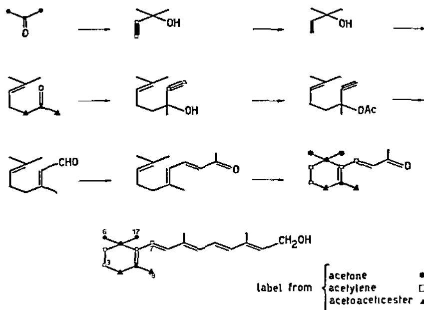
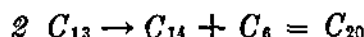


FIG 4 Labeling of the β ionone

(Wursch, 1959) The lower part of Fig 4 summarizes the labels that can be introduced during the β ionone synthesis

Engel *et al* (1953) used C^{14} labeled geraniol in a modification of this procedure Geraniol was prepared via geranic acid methyl ester from 2 methyl 2 hepten 6 one and 2 C^{14} bromoacetic acid, under the conditions of the Oppenauer oxidation in the presence of acetone, ψ ionone was obtained in one step β Ionone with a C^{14} label at the position corresponding to C 6 of the vitamin A skeleton was prepared



$$2\ C_{13} \rightarrow C_{14} + C_6 = C_{20}$$

a Synthesis of All trans Vitamin A After the investigations by Ishikawa and Matsuura on the Darzens reaction of β ionone with ethyl chloroacetate, Heilbron *et al* (1942) proved the structure of the β C_{14} aldehyde obtained

to be that of an α,β unsaturated aldehyde and suggested its usefulness for the synthesis of vitamin A (Cymerman *et al*, 1944) The realization of this vitamin A synthesis was, however, not feasible until the very low yield of the Darzens reaction was increased to over 80% in the Basle laboratories of F Hoffmann La Roche & Co Ltd by Isler *et al* (1947) Figure 5 shows the industrial manufacturing procedure for vitamin A as it was developed by the Basle group

In the first step of the synthesis, β C_{14} aldehyde is condensed with methylpent 2 en-4 yn 1 ol by a Grignard reaction Methylpentenynol is ob

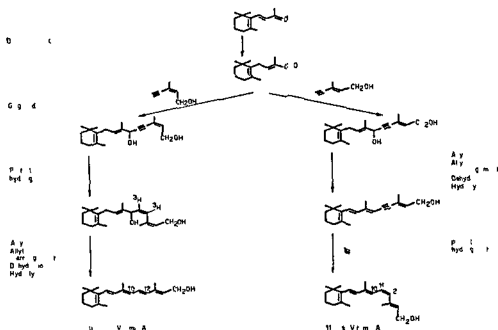


FIG 5 Synthesis of all *trans* vitamin A (left) and 11 *cis* vitamin A (right)

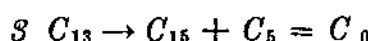
tained by condensation of methyl vinyl ketone with acetylene as a mixture of two stereoisomers, the low boiling *cis* isomer as shown in Fig 5 and the higher boiling *trans* isomer, which represents only a small fraction The structure of these isomers was proved by Oroshnik (1956), whose findings were later verified by the nuclear magnetic resonance studies by Kofler and von Planta (1959) of the isomers and the crystalline intermediates of the synthesis Partial hydrogenation of the condensation product with a highly selective catalyst (Lindlar, 1952), acylation of the primary hydroxyl group and dehydration, yields pure crystalline vitamin A acetate from which vitamin A can be prepared by saponification The rearrangement of the 11,12 and 13 14 *cis* bonds seems to occur during the dehydration stage of the synthesis

The possible radioactive labels that can be introduced are indicated in Fig 5, they are 10 C^{14}, 11,12 C^{14}, or 11,12 H^3-vitamin A 6,7 C^{14} Vitamin A (7 4 μc /mg) and 11,12 H^3 vitamin A acetate (300 μc /mg) have been prepared (Wursch, 1959)

When the corresponding 2 methylalkoxypent 2 en 4 yne compounds are used, this reaction sequence can be applied also to the synthesis of vitamin A ethers (Isler *et al*, 1949) Milas and associates (Milas, 1947, Milas *et al*, 1948), who formulated β C_{14} aldehyde as a β,γ unsaturated aldehyde, prepared crude vitamin A ethers according to this procedure It is of interest to note that, while vitamin A methyl ether has the same biological activity as the alcohol, the butyl and phenyl ethers are rather low in activity (Isler *et al*, 1949)

b Synthesis of 11-cis and 11,13 di cis Vitamin A Oroshnik (1956) showed that, with a few modifications, the Roche vitamin A synthesis can also be used for the preparation of the two known "hindered *cis*" isomers of vitamin A In a variation of the normal procedure, the condensation product of β C_{14} aldehyde and 2 methylpent 2 en-4 yn ol is acylated, dehydrated to the 11,12 dehydrovitamin A acetate, and hydrolyzed Hydrogenation of the 11 dehydrovitamin A gives the desired vitamin A isomer Figure 5 illustrates the synthesis of 11 *cis* vitamin A with *trans* methyl pentenynol, and 11,13 di *cis* vitamin A is obtained when the *cis* isomer is used

11,13 Di *cis* vitamin A can be prepared with the same labels as all *trans* vitamin A, in the case of 11 *cis* vitamin A, however, only a 10 C^{14} and 11,12 H^3 label seems feasible



$$3\ C_{13} \rightarrow C_{15} + C_5 = C_{20}$$

a Vitamin A Acid via Vinyl β ionol A very ingenious application of the Wittig reaction to the synthesis of vitamin A was made in the laboratories of the Badische Anilin und Sodafabrik (BASF), Ludwigshafen (Pommer and Sarnecki, 1958) (Fig 6) β Ionone is condensed with acetylene to dehydro β ionol, from which crystalline (β ionylidene ethyl) triphenylphosphonium halides are obtained by successive partial hydrogenation and reaction with triphenylphosphonium halides Treatment with base gives the β C_{15} phosphorane which is condensed with β formylcrotonic acid esters Saponification of the reaction product gives vitamin A acid as a mixture of the all *trans* and 13 *cis* isomers Pure vitamin A acid can, however, be prepared by iodine isomerization Vitamin A is obtained by esterification of the acid and reduction with lithium aluminum hydride

A 10,11 C^{14} and 10,11 H^3 can be introduced during the synthesis, 6,7-C^{14} vitamin A acid (20 μc /mg) was prepared according to this procedure (Wursch, 1959)

b Vitamin A Acid Isomers via β Ionylideneacetaldehyde (β C_{15} *Aldehyde*) β Ionylideneacetaldehyde can be obtained in a number of ways (see Heilbron and Weedon, 1958) The synthesis best suited for our purposes is the one by Huisman *et al* (1956) (Fig 7)

Reformatzkii reaction of β ionone and ethyl bromoacetate followed by dehydration gives predominantly *retro* C_{15} acid from which β C_{15} acid can be prepared via the β C_{15} acid chloride One obtains a mixture of the *cis trans* isomers The predominant *trans* acid can be separated from a small amount of the *cis* acid by fractional crystallization Reduction with lithium

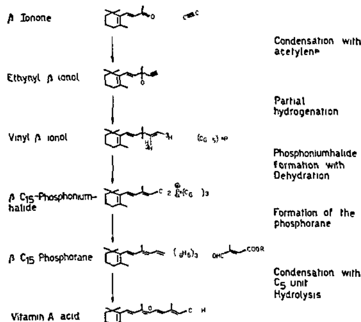


FIG 6 Vitamin A acid via vinyl β ionol

aluminum hydride and oxidation with manganese dioxide yields the corresponding aldehydes

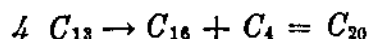
The first synthesis of vitamin A by Kuhn and Morris (1937) was achieved by condensation of β C_{15} aldehyde and β β dimethylacrolein to vitamin A aldehyde Matsui *et al* (1958) extended this approach to esters of the corresponding acid β C_{15} Aldehyde easily condenses with ethylsenecioate in liquid ammonia in the presence of alkali amides Matsui *et al* (1958) found that the use of potassium amide leads to *trans* configuration at the 13,14 double bond of the vitamin A acid, and lithium amide and sodium amide yield the 13 *cis* isomers Employing either *trans* or *cis* β C_{15} aldehyde for the condensation all the known ' unhindered *cis*" isomers of vitamin A were prepared by lithium aluminum hydride reduction of the

acids obtained Although the yields are not as high as claimed by the authors, this reaction sequence is, in view of its simplicity, preferable to the earlier synthesis of Robeson *et al* (1955) The American authors used methyl β methylglutaconate in a very similar condensation with β C_{16} aldehyde

The following labels can be introduced into the vitamin A molecule 10 C^{14}, 12,20 C^{14}, 13 C^{14}, and 15 C^{14} in the case of the all *trans* and 13 *cis* isomers Owing to rather low yields in the preparation of *cis* β C_{15} aldehyde,

FIG 7 Synthesis of vitamin A acid isomers

its labeling does not seem to be possible, 9 *cis*- and 9,13 di *cis* vitamin A can therefore be labeled only at the positions indicated in Fig 7



$$4\ \ C_{13} \rightarrow C_{16} + C_4 = C_{20}$$

A different approach to the synthesis of vitamin A from β ionone has been accomplished in the laboratories of the Distillation Products Industries, Ltd , Rochester, New York (Humphlett and Burness 1954) (Fig 8)

The Reformatzkii reaction of β ionone and propargylbromide gives the β C_{16} acetylenecarbinol, which is condensed with 4,4 dialkoxy 2 butanone The acetylenic carbinol had earlier been prepared in the laboratories of Hoffmann La Roche in Basle (1946) for an analogous approach to the

preparation of vitamin A ethers, using 4 alkoxy 2 butanone The synthesis seems to work much better in the case of the aldehyde 4,4-Dialkoxy 2 butanone is prepared in good yield by condensation of acetone with excess methyl formate (Fletscher and Hull, 1956) thus allowing the introduction of 14,20 C^{14} and 13 C^{14} labels into the vitamin A molecule Hydrogenation (11,12 H^3 vitamin A) and dehydration lead to vitamin A aldehyde enol ether, which is subsequently hydrolyzed to a mixture of *retro* , *cis* , and *trans* vitamin A aldehydes According to Benton and Robeson (1954), the two isomers indicated in Fig 8 can be separated from the mixture by com-

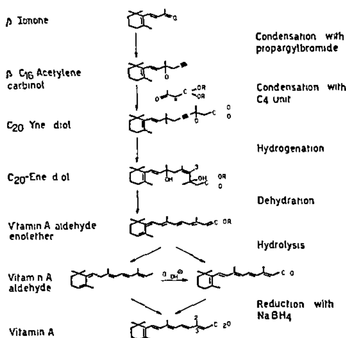
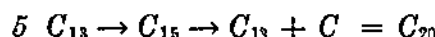


FIG 8 Vitamin A via β C_{16} acetylenecarbinol

plexing with hydroquinones Reduction of the crystalline hydroquinone complex with sodium borohydride yields vitamin A



$$5\ C_{13} \rightarrow C_{15} \rightarrow C_{13} + C\ = C_{20}$$

The synthesis of vitamin A via β C_{18} ketone has been widely used (Cawley *et al* , 1951, Wendler *et al* , 1951, Huisman *et al* , 1956) (Fig 9)

Condensation of β C_{16} aldehyde with acetone gives the β C_{18} ketone, a 12,20 C^{14} 13 C^{14} label of vitamin A therefore seems feasible

Reformatzkiĭ reaction with ethyl bromoacetate followed by dehydration, rearrangement via the acid chloride, and saponification to vitamin A acid allows the introduction of C^{14} labels at the C-14 and C 15 positions of the

vitamin A skeleton Subsequent reduction of the re esterified acid with lithium aluminum hydride gives vitamin A Wolf *et al* (1954) prepared 14 C^{14} vitamin A according to this procedure

β C_{18} Ketone had earlier been synthesized by van Dorp and Arens (1946b) via β C_{17} acid, from which the all *trans* and a small amount of a *cis* isomer could be isolated The corresponding β C_{18} ketones were prepared by treatment with methyl lithium It was later established by Graham *et al* (1949) that the *cis* β C_{18} ketone gives rise to 9 *cis* vitamin A Using β C_{18} ketone, which had been obtained by the Arens van Dorp

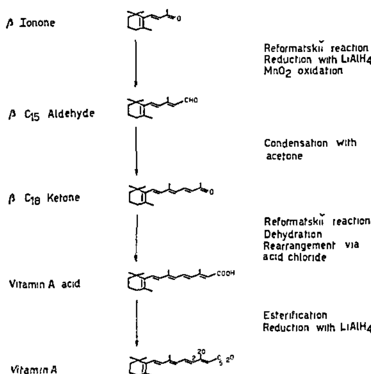


FIG 9 Vitamin A from β C_{18} ketone

method, Garbers (1956) isolated the all *trans*, 9 *cis*, and 13 *cis* C^{14} vitamin A isomers on reaction with methyl bromo(2 C^{14})acetate

IV SYNTHESES OF VITAMIN A_2

The now well established structure of vitamin A was first considered by Gillam *et al* (1938) and later by Gray (1939), Gray and Cawley (1940) and Morton *et al* (1947) The structure was finally confirmed by Farrar



Vitamin A_2

et al (1951) by reaction of vitamin A acid with *N* bromosuccinimide, followed by dehydrobromination and lithium aluminum hydride reduction (Fig 10) The alcohol obtained was identical with vitamin A Later the same authors (Henbest *et al*, 1955) extended the dehydrobromination procedure to vitamin A aldehyde Vitamin A_2 was obtained via retinene$_2$, which occurs in small amounts in the retina of freshwater fish The latter process gave the better yields

Although attempts have been made (Farrar *et al*, 1952), no synthesis of vitamin A from compounds that did not already possess the 20 carbon atoms of the vitamin A skeleton has so far been reported It appears however, that starting with the corresponding dehydro compounds, the syntheses chosen for labeling of vitamin A can also be used for the preparation

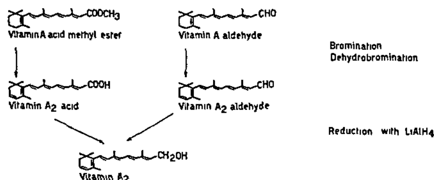
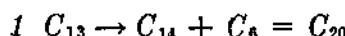


FIG 10 First syntheses of vitamin A_2 (Farrar *et al* 1951 Henbest *et al* 1955)

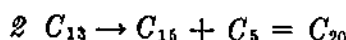
of vitamin A_2 Some syntheses of this kind have been accomplished in the laboratories of F Hoffmann La Roche in Basle



$$1\ C_{13} \rightarrow C_{14} + C_6 = C_{20}$$

In 1955 Isler *et al* succeeded in the synthesis of all *trans* vitamin A_2 from dehydro β C_{14} aldehyde (Fig 11) according to the Roche synthesis (Section III, 2 a)

Retro C_{14} aldehyde was obtained by bromination of β C_{14} aldehyde with *N* bromosuccinimide followed by dehydrobromination Saponification of its enol acetate gave dehydro β C_{14} aldehyde (Isler *et al*, 1956) from which vitamin A was obtained by the Roche procedure for vitamin A (Isler *et al*, 1957)



$$2\ C_{13} \rightarrow C_{15} + C_5 = C_{20}$$

a Vitamin A_2 via Vinyldehydro β ionol Vitamin A_2 was also prepared in the Basle laboratories of F Hoffmann La Roche & Co Ltd (1960) from dehydro β ionone by the BASF synthesis (Section III, 3, a) as shown in Fig 12

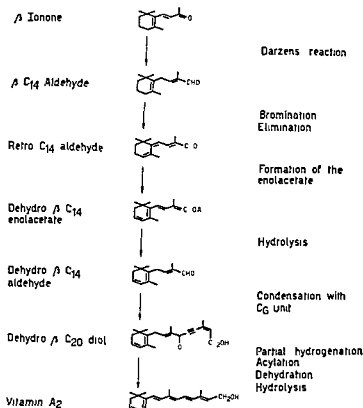


FIG 11 Vitamin A_2 from dehydro β C_{14} aldehyde

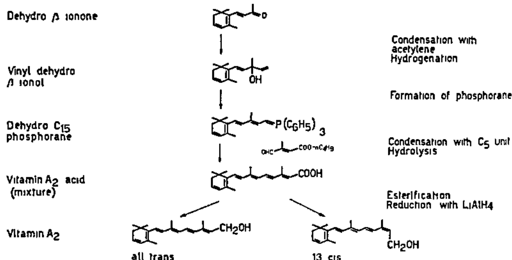


FIG 12 Synthesis of vitamin A_2 isomers from dehydro β ionone

Dehydro β ionone was prepared from β ionone according to Henbest (1951) Its condensation product with acetylene was partially hydrogenated and reacted with triphenylphosphonium bromide The dehydro β C_{15} phosphorane was prepared by addition of base and reacted with *n* butyl β

formylcrotonate After saponification the crystalline vitamin A_2 acid obtained was apparently a mixture of isomers that could not be separated by crystallization Separation succeeded, however, on reduction to the vitamin A alcohol as on chromatography of the mixture obtained, all *trans* vitamin A_2 and a hitherto unknown crystalline isomer were obtained Nuclear magnetic resonance studies indicated a 13 *cis* vitamin A_2 structure It therefore seemed desirable to check these results by a stereospecific synthesis of the 13 *cis* isomer according to the Matsui synthesis of 13 *cis* vitamin A (Section III, 3, b)

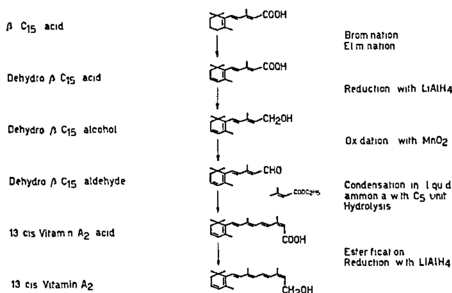


FIG 13 13 *cis* Vitamin A_2 from dehydro β ionylideneacetaldehyde

b 13 cis Vitamin A_2 from Dehydro β ionylideneacetaldehyde Dehydro β ionylideneacetaldehyde was prepared from β C_{15} acid as shown in Fig 13

The usual bromination dehydrobromination sequence gave the crystalline dehydro β C_{15} acid from *trans* β C_{15} acid Subsequent reduction with lithium aluminum hydride and manganese dioxide oxidation yielded dehydro β C_{15} aldehyde which was condensed with ethyl senecioate in liquid ammonia in the presence of sodium amide A new crystalline vitamin A_2 acid, presumably 13 *cis* was obtained Lithium aluminum hydride reduction of its methyl ester gave the same crystalline vitamin A isomer which had been obtained as a minor product by the BASF procedure (Section IV, 2, a) It therefore seems to be certain that the new crystalline vitamin A_2 has a 13 *cis* configuration, the 9 *cis* structure being excluded by its nuclear magnetic resonance spectrum (F Hoffmann La Roche & Co Ltd , 1960)

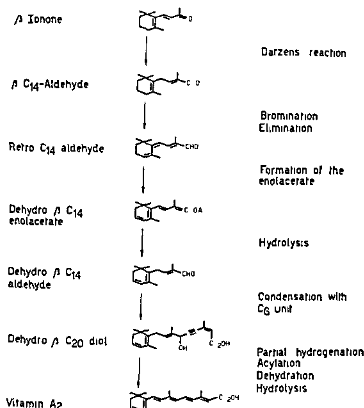


Fig 11 Vitamin A_2 from dehydro β C_{14} aldehyde

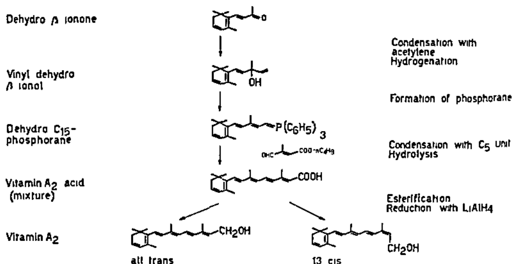


Fig 12 Synthesis of vitamin A_2 isomers from dehydro β ionone

Dehydro β ionone was prepared from β ionone according to Henbest (1951) Its condensation product with acetylene was partially hydrogenated and reacted with triphenylphosphonium bromide The dehydro β C_{15} phosphorane was prepared by addition of base and reacted with *n* butyl β

formylcrotonate After saponification the crystalline vitamin A_2 acid obtained was apparently a mixture of isomers that could not be separated by crystallization Separation succeeded, however, on reduction to the vitamin A alcohol as, on chromatography of the mixture obtained, all *trans* vitamin A_2 and a hitherto unknown crystalline isomer were obtained Nuclear magnetic resonance studies indicated a 13 *cis* vitamin A_2 structure It therefore seemed desirable to check these results by a stereospecific synthesis of the 13 *cis* isomer according to the Matsui synthesis of 13 *cis* vitamin A (Section III, 3, b)

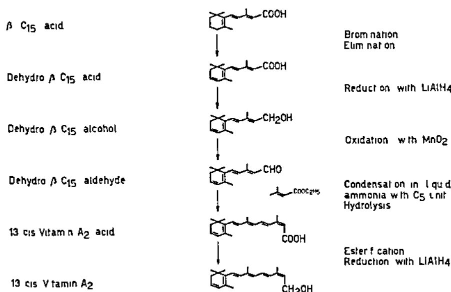


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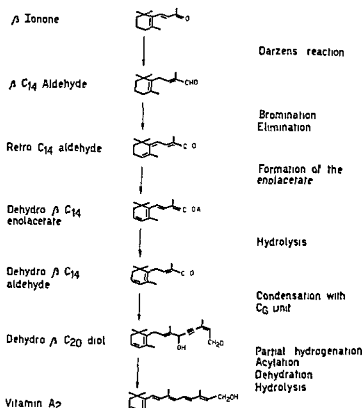


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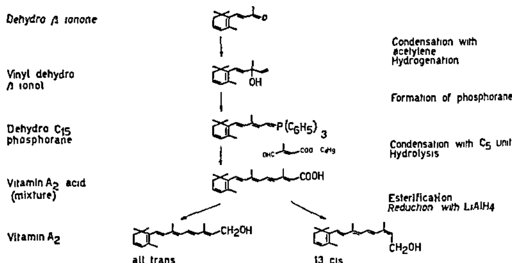


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The β apocarotenals are now available by total synthesis (Ruegg *et al*, 1959) Introduction of 15,15′ C^{14} and 15,15′ H^3 labels into the skeleton of the three compounds as indicated in Fig 14 should be possible

In the first step of the synthesis 1,1 diethoxy 2 methyl 2 penten 4 yne (C_6 acetal) is prepared by condensation of methylmalonaldehyde enol ether with acetylene and treatment with ethyl orthoformate Grignard reaction

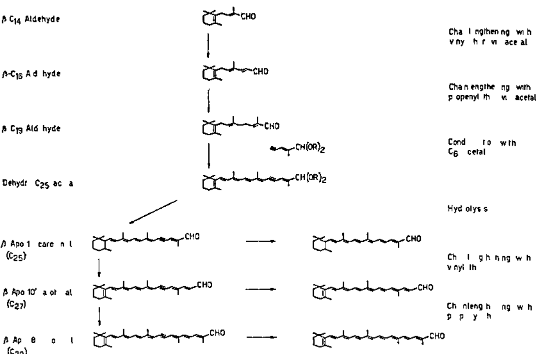


Fig 14 Synthesis of β apocarotenals from β C_{19} aldehyde

with β C_{19} aldehyde—an intermediate of the commercial β carotene synthesis—followed by dehydration and hydrolysis yields the 15,15′ dehydro apocarotenal, which on partial hydrogenation gives β apo 12′ carotenal (C $_5$) The higher vinylogs are obtained by subsequent enol ether condensation of the 15 15′ dehydroacetals with vinyl and propenyl ether followed by dehydration and partial hydrogenation

VI Concluding Remarks

This paper has indicated the research that is necessary for a fuller understanding of the functions of vitamin A in the body It seems that almost thirty years of work on synthesis have provided the chemist with the knowledge required for the labeling of vitamin A, vitamin A aldehyde, vitamin

Note added in proof

In the meantime the synthesis of the four unhindered *cis* isomers of vitamin A_2 has been accomplished by Schwieter in the Basle laboratories of F Hoffmann La Roche & Co Ltd (Schwieter 1960)

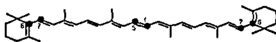
Stereoisomers of Vitamin A_2 Compounds

	x CH_2OH mp	x-CH_2-O CO-C_6H_4-N=N-C_6H_5 mp	x CHO mp	x COOH mp
all trans	17–19	74 76	77 78	183 184
13 cis	73 74	96 98	—	154 156
9 cis	77 79	83 85	54 56	159 161
9 13 dicis	below 30	81 83	—	120 122

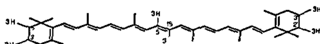
The isomers were obtained from *cis* and *trans* dehydro β C_{18} aldehyde according to the Matsui synthesis (Section III, 3 b) All *trans* vitamin A_2 alcohol has finally been crystallized

V Provitamin A Compounds

The role of β carotene and the β apocarotenals as provitamin A compounds has already been mentioned and suitable labeling of the latter compounds would obviously be a great help in solving the problem of the degradation mechanism of β carotene in the animal body



labeling with C^{14}



labeling with H^3

Aside from the labeled β carotene already prepared, namely 6,6′ C^{14} (Wursch and Schwieter, 1956) and 15,15′ C^{14} (Inhoffen *et al*, 1955), the 6,7,6′,7′ C^{14}, 2,2′,3,3′ H^3-, and 15,15′ H^3 β carotenes seem to be feasible

of synthesis according to the routes outlined by Isler and Zeller (1957) in their review of the carotenoids

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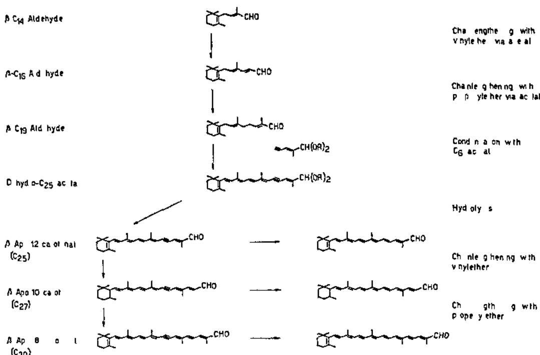


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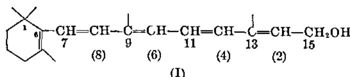
Physicochemical Assay of Vitamin A and Related Compounds

MAX KOFLER AND SAUL H RUBIN

Research Departments of F Hoffmann La Roche & Co Ltd Basle Switzerland and Nutley New Jersey

I INTRODUCTION

Vitamin A has structure (I) (Karrer *et al*, 1931)



The numbering may begin either at the end of the side chain (Geneva Nomenclature, indicated in brackets in the formula) or in the ring (this system is usual in carotenoid chemistry and will be used in the following discussion)

Since the substance designated as vitamin A_2 has been isolated from liver oils of freshwater fish, vitamin A should be called A_1 This term will be used only when the need arises to distinguish between the two substances

Sixteen *cis trans* isomers are possible in a molecule with four double bonds in the side chain, six of these isomers have been prepared to date in a pure state

This review will deal only with principles of analysis of vitamin A and of related substances Detailed procedures have recently been described by Embree *et al* (1957) as well as by Tiews (1958, 1959)

TABLE I
UV ABSORPTION MAXIMA OF VITAMIN A ALCOHOL

Solvent	λ_{max} (mμ)	$E^{1\%}_{1\,cm}$	ϵ
Ethanol	324	1800	51560
	324–325	1832	52480
Isopropanol	325 5	1835	52570
	324–326	1826	52300
Cyclohexane	326 5	1745	49900
	326–327	1737	49760
Petroleum ether	324 5	1830	52420
	324–326	1815	52000

[a] In each case the values in the first lines are by Cama *et al* (1951) and those in the second lines by Boldingh *et al* (1951)

II Determination of All *trans* Vitamin A

The all *trans* form of vitamin A is the one most widely found among the *cis trans* isomers It has the highest biological activity and is used as the International Standard This form will be referred to as "vitamin A" in the following, unless otherwise specified

1 *Determination Based on the Ultraviolet (UV) Absorption*

a The Spectrum The five conjugated double bonds in the vitamin A molecule determine the position of the absorption maximum near 325 mμ The exact position of the maxima are given for vitamin A alcohol and acetate in Tables I and II

Other esters, for instance the palmitate, have probably the same λ_{max} and ϵ values as the acetate, provided no other chromophores are present It should be noted that the molecular extinction of the esters in isopropanol

is lower than that of the free alcohols by about 4% This is of importance for calculation of analyses using saponification

b Irrelevant Absorption and the Morton Stubbs Correction Contaminants in vitamin A materials may cause an increase of extinction at the maximum of vitamin A In order to account for this so called irrelevant absorption, Morton and Stubbs (1946) developed a correction procedure based on the assumption that the irrelevant absorption is linear in the vicinity of the absorption maximum of vitamin A When this is the case, it is possible to account for the irrelevant absorption by measuring the absorption of vitamin A at three wavelengths The correction formulas have been given by

TABLE II

UV ABSORPTION MAXIMA OF VITAMIN A ACETATE

Solvent	λ_m (mμ)	$E^{1\%}_{1\,cm}$	ϵ
Ethanol	326	1550	50920
	325–326	1560	51180
Isopropanol	326	1535	50420
	324–326	1530	50280
Cyclohexane	328	1515	49770
	327–328	1520	50000
Petroleum ether	325	1595	52400
	324–326	1590	52200

In each case the values in the first lines are by Cama *et al* (1951) and those in the second lines by Boldingh *et al* (1951)

Cama *et al* (1951) for vitamin A alcohol and acetate for a series of solvents and for different "fixation points" This procedure has been adopted in several pharmacopeias (see Table III)

E_1, E_2, and E_3 designate extinctions measured at corresponding fixation points The assumption of linearity of the irrelevant absorption in the region λ_1 to λ_3 is less accurate as the interval becomes larger On the other hand, accuracy of measurement becomes more critical, the smaller the interval For this reason the reproducibility of E_{corr} is considerably less than that of the uncorrected extinction Hence it is sometimes found that E_{corr} is larger than the uncorrected extinction According to United States Pharmacopoeia (U S P) XVI, the *uncorrected* value E is used when

$$\frac{E_{corr}}{1\,030} < E < \frac{E_{corr}}{0\,970}$$

The condition that the irrelevant absorption must be linear in the region λ_1 to λ_3 is not fulfilled with compounds having an absorption maximum in this region The *cis trans* isomers of vitamin A have their maxima between the fixation points of $\lambda = 310$ mμ and $\lambda = 334$ mμ which are usually chosen, making this the region where the Morton Stubbs correction cannot be applied properly The procedure obviously does not take into acount the biological activity, which cannot be neglected in case the irrelevant absorption originates in part, for instance, from vitamin A_2

A large irrelevant absorption usually has the effect of shifting the maximum of the absorption band from its position with pure vitamin A and of widening the shape of the absorption curve so that the relative extinction

TABLE III

CORRECTION FORMULAS[a]

	Solvent	Fixation points			E_c
		λ_1	λ_2	λ_3	
Pharmacopoeia internationalis	Cyclohexane	312 5	326 5	336 7	$7(E_2 - 0\ 422\ E_1 - 0\ 578\ E_3)$
U S P XVI and IUPAC Vit Div [c]	Isopropanol	310	325	334	$6\ 815\ E_2 - 2\ 555\ E_1 - 4\ 260\ E_3$
British Pharmacopoeia 1958	Cyclohexane	312 5	327 5	337 5	$7(E_2 - 0\ 405\ E_1 - 0\ 595\ E_3)$[b]
		312 5	326 5	336 5	$7(E_2 - 0\ 422\ E_1 - 0\ 578\ E_3)$

[a] The data are for free vitamin A alcohol unless stated otherwise
[b] Ester
[c] IUPAC Vit Div = International Union of Pure and Applied Chemistry Vitamin Assay Division

E/E_{max}, where E_{max} is the extinction at the maximum of the vitamin A absorption curve, is higher than with pure vitamin A [According to U S P XVI and the recommendations of the Vitamin Assay Division of the International Union of Pure and Applied Chemistry (IUPAC) (Brunius, 1958), the measurement of the absorption of vitamin A is always made in isopropanol, in that case E_{max} means E_{325}] In case the irrelevant absorption is high, purification of the material by chromatography is recommended The Vitamin Assay Division of IUPAC recommends chromatographic purification of the unsaponifiable fraction when the maximum is found outside the region of 323–327 mμ, or when E_{300}/E_{325} is larger than 0 73 (Brunius, 1958)

c Other Methods for Removing Irrelevant Absorption Procedures have been described whereby the extinction at the maximum of vitamin A absorption is measured before and after destruction of the vitamin These procedures

are based on the premise that vitamin A is completely destroyed, but not the accompanying substances that absorb at 325 mμ The destruction of vitamin A has been carried out by irradiation (Bessey *et al*, 1946) or by reaction with sulfuric acid (Fox and Mueller, 1950)

d The International Unit (I U) and the Conversion Factor One international unit is equal by definition (World Health Organization, 1950) to 0 344 μg of pure all *trans* vitamin A acetate (corresponding to 0 300 μg vitamin A alcohol) One gram of vitamin A acetate contains accordingly 2,907,000 I U, and 1 gm of vitamin A alcohol 3,333,000 I U The conversion factor *f* for calculation of the biological activity from UV measurement (Table IV) is defined by

TABLE IV

CONVERSION FACTORS FOR VITAMIN A ALCOHOL AND ACETATE

Solvent	Vitamin A alcohol	Vitamin A acetate
Ethanol	1850	1880
	1820	1860
Isopropanol	1820	1895
	1825	1900
Cyclohexane	1910	1920
	1920	1910
Petroleum ether	1820	1820
	1835	1830

$$f = \frac{\text{I U/gm}}{E_{1\text{cm.}}^{1\%}\ (\text{max})}$$

The values of *f* follow from Tables I and II The Vitamin Division of IUPAC recommends the value $f = 1830$ for vitamin A alcohol in isopropanol (Brunius, 1958)

2 Procedures Based on Light Absorption of Vitamin A Derivatives

These procedures are based mostly on color reactions, i e reactions in which derivatives are formed which absorb in the visible The intensity of the color so produced is measured in a colorimeter or spectrophotometer Since Beer s law does not hold with large changes in concentration, calibration curves are necessary Impurities or other accompanying substances may affect the final extinction and the rate of color development, so that an increment procedure is advisable

Reactions have been used with strong acids such as H_2SO_4, $HClO_4$, etc, with metal halogenides such as antimony trichloride, arsenic trichloride, ferric chloride, and also with glycerin dichlorohydrin These reactions are not specific for vitamin A and give positive results with many other substances, in particular with polyenes

a Reaction with Antimony Trichloride [According to Bruggemann *et al* (1952, 1952/53) the active species are traces of $SbCl_5$] This so called "Carr Price" reaction is the most widely used of the color reactions The very unstable blue color is measured in rapidly indicating colorimeters (using a filter centered around 620 mμ) or in spectrophotometers The measurements can be made in the following ways (1) The extinction is measured after a definite interval, for instance 30 seconds after the addition of the reagent (2) The highest extinction reached is recorded (3) The extinction is measured at definite intervals and extrapolated to $t = 0$

The $E_{1\,cm}^{1\%}$ values of the color in the "Carr-Price" reaction found in the literature have no physical significance since they depend essentially on the way the reaction is carried out These values can be useful, however, for comparative measurements

The use of an internal standard is recommended (Oser *et al*, 1943) in order to eliminate the effect of accompanying substances, temperature, and state of the reagent The standard curves for vitamin A alcohol and for the different esters are not identical This should be taken into consideration in the direct determination without saponification (Bruggemann *et al*, 1952/53)

b Reaction with Glycerin Dichlorohydrin (Sobel and Werbin, 1945, 1946) The color fades less rapidly in this reaction than in the reaction with antimony trichloride However, this reaction is less sensitive The reagent has to be "activated," a little understood process

c Determination by Conversion to Anhydrovitamin A Embree and Shantz (1940) have pointed out the possibility of determining vitamin A by converting it into anhydrovitamin A which has a high UV absorption This derivative, which has a very characteristic spectrum, has a maximum at longer wavelength than vitamin A and usually shows less irrelevant absorption than vitamin A A detailed procedure has been described by Budowski and Bondi (1957)

3 Fluorometric and Other Methods

The yellowish green fluorescence of vitamin A and of its esters is sometimes used for qualitative identification, particularly in chromatographic work The vitamin A esters are converted by irradiation into unstable compounds that show an intense green fluorescence in addition to the characteristic fluorescence of the vitamin A substance (Sobotka *et al*, 1943) Fluorometric methods have not found their way into quantitative deter-

mination of the vitamin The same is true for the polarographic method (Keller and Weiss, 1955/56)

4 Separation Procedures

Separation methods are used, on the one hand, in order to separate vitamin A from substances that interfere with the analysis (irrelevant absorption inhibitors in color reactions) and, on the other hand, to separate different substances that have vitamin A activity

a Saponification Saponification helps to diminish considerably irrelevant absorption as well as inhibition effects in the Carr Price reaction It also provides a way to carry out a uniform spectrophotometric determination of vitamin A alcohol

b Adsorption Chromatography Foremost among the separation procedures is adsorption chromatography with neutral to alkaline Al_2O_3 Secondary

TABLE V

Cis trans Isomers of Vitamin A

Trivial name (Wald)	Numbering according to Geneva System	Numbering usual in carotenoid chemistry
—	all *trans*	all *trans*
Neo a	2 *cis*	13 *cis*
Neo b	4 *cis*	11 *cis*
Neo c	2 4 di *cis*	11 13 di *cis*
Iso a	6 *cis*	9 *cis*
Iso b	2 6 di *cis*	9,13 di *cis*

calcium phosphate is also used sometimes Numerous other adsorbents have been recommended for special problems but have not come into general use Adsorption chromatography also provides a means of separating vitamin A alcohol from its esters (Muller, 1944, 1947)

c Distribution Distribution between two immiscible solvents in the form of column partition chromatography provides a method for the separation of the vitamin A alcohol, acetate, and palmitate (Rindi, 1958, Wiss and Gloor, 1958) The same result can be achieved by paper chromatography (Huber 1956, Kaiser and Kagan, 1956, Suzuki and Sahashi, 1957) and thin layer chromatography (Roche Laboratories, unpublished data) Chromatographic columns are preferred for the quantitative determination because of the sensitivity of vitamin A to oxidation

III Determination of *cis trans* Isomers of Vitamin A

The known *cis trans* isomers among the sixteen that are theoretically possible are listed in Table V There are, according to Pauling, twelve

sterically hindered and four unhindered *cis trans* isomers All the unhindered isomers are known all *trans*, neo a, iso a, and iso b Neo b and neo c are hindered isomers

Methods for the quantitative isolation and determination of the isomers in mixtures would be of great interest because of differences in the biological activity of the isomers The relative biological activity of the acetates with respect to all *trans* vitamin A acetate as 100% has been determined as follows (Ames *et al*, 1955a, Ames, 1958) neo a = 75%, neo b = 24%, neo c = 15%, iso a = 21%, iso b = 24%

The determination of the composition of the mixture of isomers in thermodynamic equilibrium would require accurate methods or determination of the individual isomers In our opinion, such methods are not available at present In thermodynamic equilibrium one would expect to find in the

TABLE VI

ULTRAVIOLET ABSORPTION MAXIMA OF THE *cis trans* ISOMERS OF VITAMIN A ALCOHOL IN ETHANOL

Isomer	λ_{max} (mμ)	$E_{1\,cm}^{1\%}$	ϵ	References
All *trans*	325	1832	52480	Boldingh *et al* 1951
Neo a	328	1686	48300	Robeson *et al* 1955a
Neo b	319	1220	39400	Brown and Wald 1956
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Iso a	323	1477	42300	Robeson *et al* 1955a
Iso b	324	1379	39500	Robeson *et al*, 1955a

first place the four unhindered isomers the free energies of which are lower than those of the hindered isomers It should be possible to reach equilibrium starting from each of the four isomers Catalysts, such as iodine (Hubbard, 1956), can hasten the establishment of the equilibrium (*irradiation usually favors high energy isomers) There are no exact data on the kinetics* of isomerization in commercial or physiological preparations since reliable analytical procedures do not exist

1 The Ultraviolet Absorption Spectrum

It is apparent from Table VI that the UV absorption maxima of the isomers are so near to one another that simultaneous determination of individual isomers, at least the unhindered isomers, is not possible

Iso a and iso b show, in addition to the main maximum given in the table, a "*cis* peak" at about 260 mμ, neo c has a secondary maximum near 235 mμ The secondary maximum can be of assistance in the analysis of mixtures of pure isomers

For the corresponding values of the acetates see Table VII

It is wrong in principle to use the Morton Stubbs correction for the determination of all *trans* vitamin A in a mixture of isomers, since the condition of linearity of the irrelevant absorption is not fulfilled However, in the case of a mixture containing only all *trans* and neo a vitamin A, the use of this procedure accidentally yields an almost correct answer

2 *The Infrared Absorption Spectrum*

The infrared absorptions of all *trans*, neo a, iso a, and iso b vitamin A alcohol have been determined by Robeson *et al* (1955a) No quantitative determination can be based on the small differences found Somewhat larger

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Isomer	λ_{max} (mμ)	$E^{1\%}_{1\,cm}$	ϵ	References
All *trans*	325–326	1560	51180	Boldingh *et al* 1951
Neo a	328	1430	47000	Robeson *et al* 1955a
Neo b	320–321	973	31960	Roche Laboratories (unpubl data)
Neo c	310–311	859	28220	
Iso a	323	1200	39400	Robeson *et al* 1955a
Iso b	324	1110	36500	Robeson *et al* 1955a

differences in the infrared spectrum are shown by the isomeric aldehydes (Robeson *et al*, 1955b) which can be obtained from the alcohols by oxidation The analysis of the four sterically unhindered isomers is possible according to Brown *et al* (1959), when their concentration is at least 75% Further data on individual infrared bands of neo b and neo c vitamin A alcohol have been given by Oroshnik (1956)

3 *The Nuclear Magnetic Resonance Spectrum*

Proton resonance spectra have been obtained with five of the six isomers at an oscillator frequency of 56 4 megacycles per second and with tetramethylsilan as an internal standard [Figs 1–5 (von Planta, unpublished data)]

The "chemical shifts" are given in Table VIII The assignment of the resonance to protons of the C 13 methyl was made by comparison with the spectrum of a vitamin A analog lacking the methyl group at C 13

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Neo b	320–321	973	31960	Roche Laboratories (unpubl data)
Neo c	310–311	859	28220	
Iso a	323	1200	39400	Robeson *et al* 1955a
Iso b	324	1110	36500	Robeson *et al* 1955a

differences in the infrared spectrum are shown by the isomeric aldehydes (Robeson *et al* 1955b) which can be obtained from the alcohols by oxidation The analysis of the four sterically unhindered isomers is possible according to Brown *et al* (1959), when their concentration is at least 75% Further data on individual infrared bands of neo b and neo c vitamin A alcohol have been given by Oroshnik (1956)

3 The Nuclear Magnetic Resonance Spectrum

Proton resonance spectra have been obtained with five of the six isomers at an oscillator frequency of 56 4 megacycles per second and with tetra methylsilan as an internal standard [Figs 1–5 (von Planta unpublished data)]

The "chemical shifts" are given in Table VIII The assignment of the resonance to protons of the C 13 methyl was made by comparison with the spectrum of a vitamin A analog lacking the methyl group at C 13

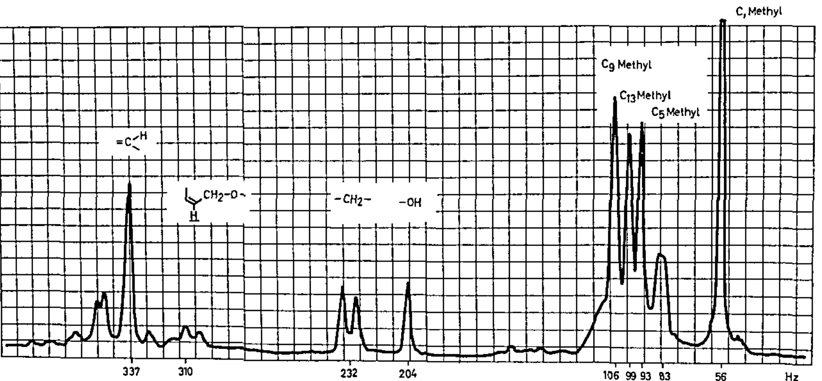
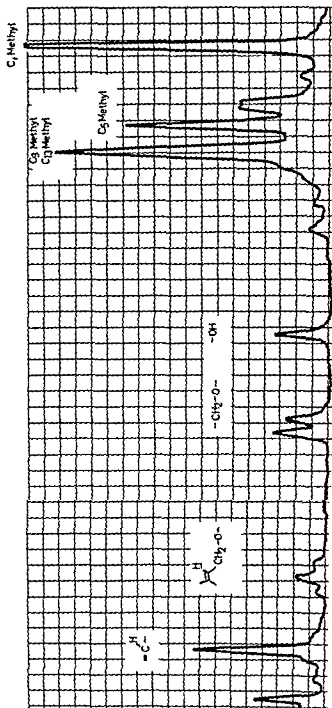


FIG 1 Proton resonance spectrum of all *trans* vitamin A



C1 Methyl
C9 Methyl
C13 Methyl
C5 Methyl
-OH
-CH2-O-

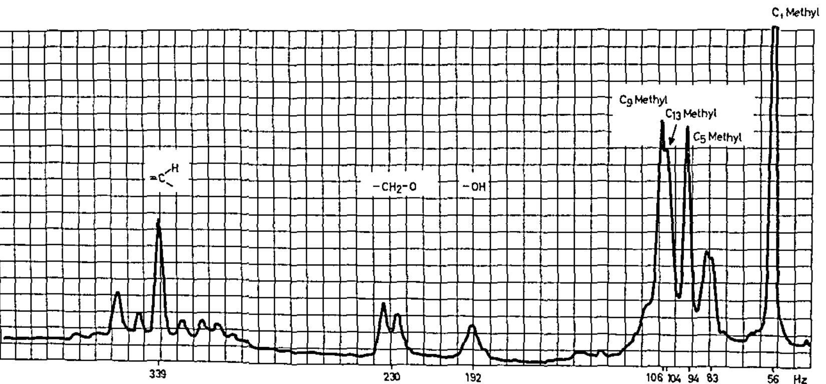


Fig 3 Proton resonance spectrum of neo b vitamin A

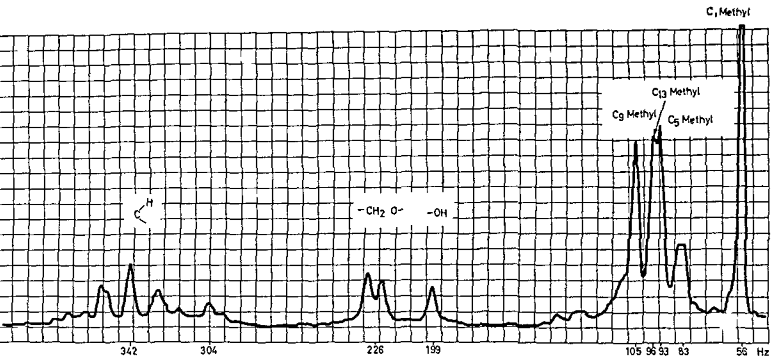


FIG 4 Proton resonance pectrum of iso a vitamin A

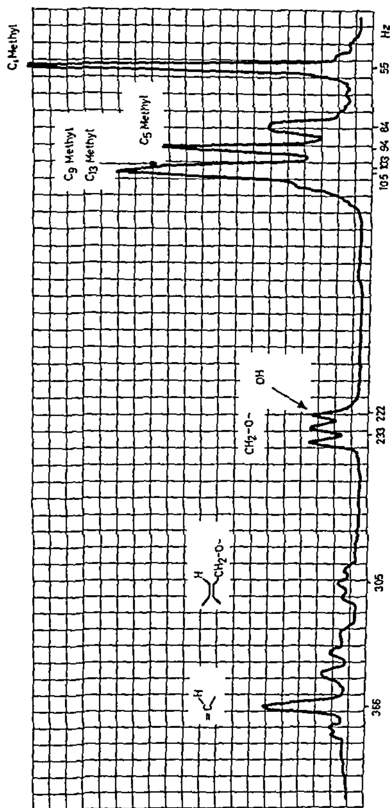


FIG 5 Proton resonance spectrum of iso b vitamin A

A quantitative nuclear magnetic resonance analysis of enriched mixtures of isomers is planned, using an electronic integrator

4 The X Ray Powder Diagram

The method is limited to crystalline substances and is capable of showing distinct differences in lattice spacings between the isomers It is useful for identification only [Figs 6–9 (von Planta, unpublished data)]

5 The Antimony Trichloride Reaction

Since this reaction does not afford any distinction between the isomers, it suggests that the same product is formed with each of the isomers Accordingly, the Carr Price reaction gives the total isomers present

TABLE VIII
CHEMICAL SHIFTS OF THE PROTONS AT THE DOUBLE BONDS AND OF THE C 13 METHYL

Isomers	=C—H (c/s)	C 13 Methyl (c/s)
All *trans*	337	99
Neo a	343	106
Neo b	339	104
Iso a	342	96
Iso b	366	103

6 The Maleic Anhydride Reaction

Maleic anhydride reacts with all *trans* vitamin A much faster than with neo a vitamin A, a property which was used by Robeson and Baxter (1947) for the determination of neo a vitamin A in mixtures with all *trans* vitamin A The formation of the adduct results in the disappearance of the absorption band at 325 mμ, and the blue color is not formed in the Carr Price reaction The recovery value R is obtained by determining the content of vitamin A after a predetermined developing time in a solution with and without the addition of maleic anhydride The percentage composition of the mixture is calculated from this value and the R values obtained under the same conditions with pure all *trans* and neo a vitamin A

It has been found that iso a, as well as all *trans* vitamin A, reacts quickly whereas the other isomers react much more slowly, however, the R values are so different within each group that only one substance of each group should be present in the analysis

ing isorhodopsin (λ_{max} = 487 mμ), whereas no reaction takes place with the other isomeric aldehydes In this way neo b- and iso a vitamin A can be determined, even when both are present together after oxidation to the respective aldehydes (Hubbard, 1955/56) Iso b vitamin A aldehyde can be isomerized quickly to iso a aldehyde by irradiation and then determined

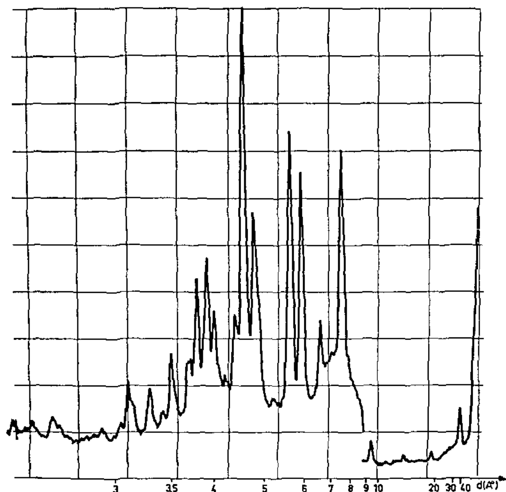


Fig 7 X Ray diffraction diagram (CuK) of neo a vitamin A

in a similar manner (Hubbard and Wald, 1952/53, Hubbard *et al* , 1952/53) The method is not suitable for routine analysis owing to the difficulty in preparing opsin

8 Separation Procedures

Only chromatographic procedures (adsorption and partition) have a chance of succeeding in analytical separations because of the close simi

larity of the isomers Neo a can be separated from all *trans* vitamin A by adsorption chromatography on secondary calcium phosphate (Bro Rasmussen *et al*, 1955) and aluminum oxide (Barnholdt, 1956) Five isomers were separated by Barnholdt and Hjarde (1957)

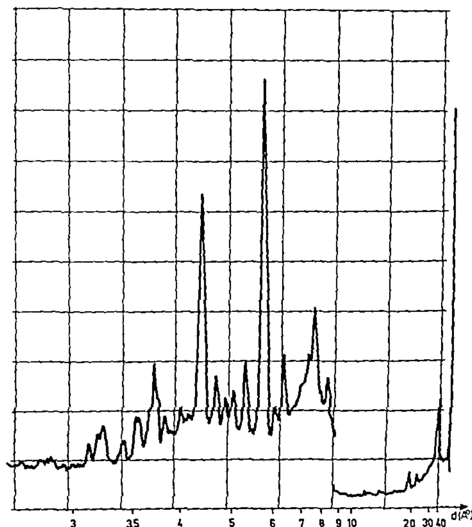
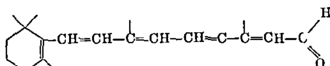


FIG 8 X Ray diffraction diagram (CuK$_\alpha$) of iso a vitamin A

We have attempted a separation of the six isomers by thin layer chromatography on silica gel, we found the rates of migration increasing in the series all *trans*, iso a, iso b, neo a, neo b, and neo c The sensitivity of vitamin A to oxidation obviates the use of this procedure in quantitative determinations

IV The Determination of Derivatives of Vitamin A and of Related Compounds

1 *Vitamin A Aldehyde (Retinene)* (II)



(II)

Fig 9 X Ray diffraction diagram (CuK) of iso b vitamin A

All six aldehydes are known, corresponding to the vitamin A alcohol isomers described above

The molar biopotencies are given with respect to all *trans* vitamin A

acetate = 100%, all *trans*, 91%, neo a, 93%, neo b, 48%, neo c, 31%, iso a, 19%, iso b, 17% (Ames *et al*, 1955b, Ames, 1958)

The absorption maxima in the ultraviolet are shifted to longer wave lengths as compared with those of vitamin A alcohol (Table IX) Secondary maxima have also be observed

The infrared absorption spectra, with the exception of neo c, have been reported by Robeson *et al*, 1955b

TABLE IX
ULTRAVIOLET ABSORPTION MAXIMA OF *cis trans* ISOMERIC ALDEHYDES IN ETHANOL

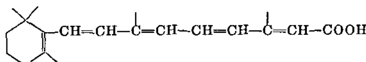
Isomer	λ_{max} (mμ)	$E^{1\%}_{1\,cm}$	ϵ	References
All *trans*	381	1530	43400	Robeson *et al* 1955b
Neo a	375	1250	35600	Robeson *et al*, 1955b
Neo b	376 5	878	24900	Brown and Wald 1956
Neo c	373	700	19900	Wald *et al* 1955
Iso a	373	1270	36100	Robeson *et al*, 1955b
Iso b	368	1140	32400	Robeson *et al* 1955b

TABLE X
ULTRAVIOLET ABSORPTION MAXIMA OF *cis trans* ISOMERIC VITAMIN A ACIDS IN ETHANOL

Isomer	λ_{max} (mμ)	$E^{1\%}_{1\,cm}$	ϵ
All *trans*	350	1500	45200
Neo a	354	1320	39800
Iso a	345	1230	36900
Iso b	346	1150	34500

The Carr-Price reaction gives a maximum at 664 mμ (Hubbard *et al*, 1952/53, Robeson *et al*, 1955b) and is the same for all isomers

2 Vitamin A Acid (III)

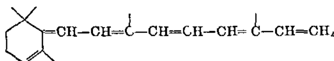


(III)

This acid appears as an intermediate in various syntheses The UV absorption spectra (Robeson *et al*, 1955a) are given in Table X The anions absorb at shorter wavelengths and more strongly

The growth promoting effect of vitamin A acid, as is the case with β carotene, is closely dependent on the method of administration (Arens and van Dorp, 1946, van Dorp and Arens, 1946) Under the conditions of U S P XIII growth test an activity of 42% was found for the all *trans* form (unpublished investigations Roche Laboratories)

3 Anhydrovitamin A

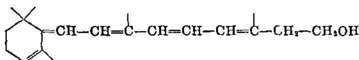


(IV)

This substance has a *retro* structure (IV) (Shantz *et al*, 1943, Meunier *et al*, 1943, Oroshnik *et al*, 1952)

It is formed by a dehydration of vitamin A and is sometimes found with the latter as an impurity Its biological activity is very low (Shantz *et al*, 1943) As expected from its structure, it is held less strongly on aluminum oxide than vitamin A alcohol and its esters The system of six conjugated double bonds determines, in ethanol, absorption maxima at 351 mμ ($E^{1\%}_{1\,cm}$ = 2500), 371 mμ ($E^{1\%}_{1\,cm}$ = 3650), and 392 mμ ($E^{1\%}_{1\,cm}$ = 3180) The Carr Price reaction gives the same absorption maximum (620 mμ) as with vitamin A, which justifies the assumption that the same product is formed (Shantz *et al*, 1943)

4 retro Vitamin A (V)

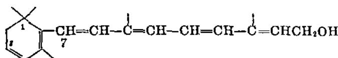


(V)

The acetate shows an absorption maximum at 348 (ε = 56800), as well as secondary maxima at 333 and 367 mμ (Beutel *et al*, 1955)

The same chromophore belongs presumably to a compound erroneously named isoanhydrovitamin A (Shantz *et al*, 1943, Oroshnik, 1954) The absorption maxima are at 332, 348, and 366 This compound is held somewhat more strongly on aluminum oxide than anhydrovitamin A, and in the Carr Price reaction shows an absorption maximum at 623 mμ

5 Vitamin A



(VI)

to map the occurrence in nature of "the fat soluble factor A," a simple test sufficed If feeding the test material to vitamin A deficient rats induced resumption of growth, a designation of "good" or "excellent" source of vitamin A activity was given, depending on whether much or little was fed Next, the needs of chemists synthesizing vitamin A, of physiologists studying its function, and of government regulatory agencies demanding label potency led to improvement in the precision and accuracy of bioassays The rigorous requirements of drug and feed manufacturing companies for exact measurement of vitamin A to assure potency guarantees and to assess inventories also helped to make bioassays more quantitative When the purchase price of billions of units of vitamin A depended on bioassay results, the smaller the limits of error (confidence or fiducial limits) of the assay the better

In the current stage of evolution of vitamin A bioassays, we realize that two types are needed One type to measure the potential or total vitamin A activity per molecule is needed to study and evaluate such items as vitamin A isomers, provitamins A, metabolites, and synthetic compounds related to vitamin A Eventually results from this type may lead to complete understanding of the relationship between chemical structure and biological activity

The other type of bioassay to measure the over all benefit of the vitamin A preparation administered is needed to evaluate the practical vitamin A potency of pharmaceuticals, foods, and feeds It is conducted under simulated practical feeding conditions, and the potential availability of the vitamin A present is affected by such factors as losses due to mixing, processing, and storing The results of such an assay, in which potency and factors affecting potency are confounded, represent practical availability

This essay will review briefly the various bioassay methods available and illustrate some of them with examples from the literature Those aspects of technique and interpretation that make the bioassays either a measure of total vitamin A or a measure of availability will be emphasized

II History

1 *Methods*

Detailed working directions for determining vitamin A activity have been compiled from time to time in monographs or review chapters that are still worth consulting to get the author's preference (or perhaps bias) for a certain method

The bioassayist's bible for many years was Coward's "Biological Standardization of the Vitamins," published in 1938 and revised in 1947 Coward did an extraordinary job with all the vitamins during this period when

vitamin research was at its peak The biological determination of vitamin A was particularly well treated, probably because Coward had worked out so many of the details in her own laboratory

Also in 1947, the chapter on vitamin A by E M Nelson and J B De Witt appeared in *Biological Symposia* (Volume XII, "Estimation of the Vitamins") and emphasized the problem of control and government regulatory laboratories

In 1951, in "Vitamin Methods," Bliss and Gyorgy, and Guerrant gave the touch of statistical masters to the subject of vitamin A bioassays and made them more quantitative

Embree and co workers in *Methods of Biochemical Analysis* (1957) reviewed the various vitamin A bioassays and gave details for planning, performing and statistically analyzing the results of (1) rat growth, (2) liver storage and (3) vaginal smear bioassays They showed strong preference for the liver storage method

What lessons have we learned from all the fine, conscientious work of the past that has brought vitamin A bioassays to their present level of development and utility? Too many to give them all here, but some of the most important would certainly be these (1) Use a good, stable reference standard (2) Employ a bioassay in which the criterion of response is specific for vitamin A (3) Always show the confidence limits as an integral part of the expression of biopotency

2 Standards

Of the many characteristics constituting the ideal reference standard for use in vitamin A bioassays, the most important is 'stability " For many years, before an exact correlation was attained between biological and physicochemical assays, almost every expression of biopotency was suspect because the reference standard used was unstable As the reference standard lost potency, any substance compared with it showed an unpredictable and higher than actual biopotency

The official standards used throughout the years are listed in Table I Changes were made primarily to replace materials of lesser with those of greater stability The ultimate of course was the replacement of a "material" standard with a "descriptive" or "specification" standard achieved for both β carotene and vitamin A in 1957 by the World Health Organization Now investigators are freed of the necessity of using a particular vitamin A standard Nevertheless, a supply of all *trans* vitamin A acetate in cottonseed oil, standardized at 100 000 U S P or International Units per gram, convenient for research purposes and for physicochemical or biological assay, is available as indicated in Table I

To meet the need for a vitamin A reference standard suitable for mixing

to map the occurrence in nature of "the fat soluble facto sufficed If feeding the test material to vitamin A defic resumption of growth, a designation of "good" or "ex vitamin A activity was given, depending on whether mucl Next, the needs of chemists synthesizing vitamin A, of ph ing its function, and of government regulatory agencies potency led to improvement in the precision and accur The rigorous requirements of drug and feed manufacturi exact measurement of vitamin A to assure potency guarant inventories also helped to make bioassays more quantita purchase price of billions of units of vitamin A depended on l the smaller the limits of error (confidence or fiducial limits) better

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in feed, as is done in bioassays using poultry and other livestock, a gelatin coated product was developed It contains 10,000 units vitamin A per gram, and collaborative bioassays under the auspices of the Animal Nutrition Research Council showed excellent stability and availability Samples of this product are distributed by the U S P as shown in Table I

III Methods

1 Based on Specific Responses

a Xerophthalmia Coward (1947) and Wagner (1953) successfully utilized the cure of xerophthalmia as a criterion of response of vitamin A activity, but most other workers have used this symptom merely as an auxiliary or confirmatory sign of vitamin A depletion The steps of the bioassay are Deprive rats of vitamin A until a definite state of inflammation of the conjunctiva of the eye develops Administer graded doses of vitamin A standard and test material daily and measure the response as the time in days for xerophthalmia to clear up Calculate the potency of the unknown in terms of the standard by comparing their parallel line, dose response curves (straight lines relating response to logarithm of dose) The precision is inherently poor, and this procedure is rarely used

The underlying dysfunction causing xerophthalmia is the decrease in resistance of the conjunctival epithelium to bacterial infection Other tissues and organs also become more susceptible to invasion by microorganisms and parasites as the animal becomes depleted of vitamin A Research to develop a new bioassay based on some other aspect of lowered resistance might be very fruitful Many years ago Boynton and Bradford (1931) inoculated rats in various stages of vitamin A deficiency with standardized suspensions of *B mucosus capsulatus* and measured mortality The resistance of the animals to the infection was inversely proportional to the length of time on the vitamin A low diet prior to inoculation More recently, an experiment of Erasmus *et al* (1960) showed that mortality rate in poultry injected with a standardized culture of coccidia is related inversely to the vitamin A intake The fact that chickens naturally infected with coccidia have very little if any liver vitamin A compared with unaffected birds in the same flock was reported by Davies in 1952 He also found that vitamin A administration accelerates recovery from coccidiosis Another possibility for a bioassay is based on the response of rats to standard injections of foreign materials, e g turpentine or almond oil to produce sterile abcesses Both blood and liver vitamin A levels are significantly reduced (Kagan and Kaiser, 1955) If the procedure could be made quantitative it might be a measure of the total effective or mobilizable vitamin A in the body

TABLE I

VITAMIN A REFERENCE STANDARDS

Date	Material	Standard unit (μg)	Authority
	Provitamin Standard		
1930-1934	Crystalline carotene	1 0	League of Natio Permanent Co mission on Biol ical Standard tion (Hume Chick, 1935)
1935-1956	Crystalline β carotene [300 μg (500 I U)/gm in coconut oil, since 1949 in cottonseed oil]	0 6	
1956-present	No material standard pure β carotene commercially avail able for use if needed Express potency as I U of provitamin A	0 6	I U P A C (19
	Pharmaceutical Standard		
1934-1939	Cod liver oil No 1 (3000 units per gram), secondary refer ence standard		U S P X
1939-1948	Cod liver oil No 2 and 3 (1700 units per gram), secondary reference standards		U S P XI
1948-1957	Vitamin A acetate in cottonseed oil (10 000 units per gram) [in gelatin capsules contents 250 ± 1 mg]	0 344	W H O
1957-present[b]	*Vitamin A (trans) acetate in* cottonseed oil (100 000 units per gram) [in gelatin capsules contents 250 + 1 mg]	*0 344*	U S P macc natio W H
	Feed Standard		
1959-present[b]	Vitamin A (*trans*) acetate in gelatin beads (10 000 units per gram)	0 344	A N

[a] The International B ological Standard for Provitamin A was discontinued in 1956 (

[b] Currently available from U S Pharmacopoeia Reference Standards 46 Park Av U S A

Currently available from Centre for Authentic Chemical Substances Apotek r Stockholm Sweden

erence standard The dose response curve is a straight line function between logarithm of dose and response in days The assay slope is

$$b = \frac{1}{0.3010} \frac{T_3 - T_1 + T_3 - T_{1u}}{N_1 - N_{3r} + N_{1u} + N_{3u}}$$

where T_{1r}, T_{2r}, and T_3 = total response in days for each reference standard and N_1, N_2, and N_3 = number of animals in each reference standard group, T_{1u}, etc, and N_{1u}, etc, represent response and numbers of animals in the groups supplemented with the unknown material

The relative biopotency is

$$\mathrm{RP} = \mathrm{antilog}\,\frac{1}{b}\left(\frac{T_u}{N_u} - \frac{T}{N_r}\right)$$

where T and T_u are total responses in days for the reference and the unknown, respectively, and N_r and N_u are total numbers of animals supplemented with reference and unknown, respectively

The standard error of the relative potency and confidence limits can be calculated (Embree *et al*, 1957) For this type of bioassay, the approximate 95% confidence limits are ± 12%

d Tissue Content (*1*) *Blood levels* Bioassays based on the chemical determination of vitamin A in blood have a place in the repertory of the nutritionist but the results are usually of less value than comparable determinations in another tissue—liver, the principal storage site in the body for vitamin A

The fasting blood level of vitamin A is constant and characteristic for each individual animal, including man It does not change significantly in response to changes in vitamin A intake unless the dose is massive Administration of doses of 7000 I U per kilogram body weight or more overwhelms the homeostatic mechanism and results in a temporary increase in plasma vitamin A The curve relating blood vitamin A concentration to time is termed "tolerance curve" although "absorption curve" would be better Its maximum occurs at 4–7 hours and it returns to the original predose level at 24 hours The area under the curve is considered proportional to the amount of vitamin A absorbed from the intestinal tract (Chesney and McCoord, 1934)

Proponents of this vitamin A absorption test as an assay suitable for comparing the potency of a variety of vitamin A products claim good accuracy and high precision for it (Lewis *et al*, 1947, Lewis and Cohlan, 1950 Week and Sevigne 1950) Other investigators find considerable variability, interpret the results as a measure of rate rather than amount of absorption and conclude that its best use is in the study of malabsorp

b *Nyctalopia* (Night Blindness) Many attempts have been made to this phenomenon as the basis for a bioassay both *in man and in experime* animals Since the fundamental reaction involved, the formation of rhod *sin from vitamin A aldehyde* and opsin in the rods of the retina, is the known specific function of vitamin A, such efforts were justified Howe the relative difficulty of measuring night blindness and the fact that is a late symptom—almost the last to appear in the course of developm of A deficiency—combine to make this bioassay of little practical use

c *Cornification of Vaginal Epithelium One of the earliest* disco symptoms of vitamin A deficiency in the female rat was sterility due interruption of the normal estrous cycle (Evans and Bishop 1922) Vag smears show persistent cornification (large, thin, nonnucleated, scale keratinized cells) in vitamin A deficiency, when sufficient vitamin A is ministered as a supplement, the normal estrous cycle is resumed Ma investigators, particularly Mason and Ellison (1935), Pugsley and workers *(1944), and Clarke and Todd (1957),* contributed modificatio and the technique is used routinely by some groups with excellent resul For example, scientists of the Canadian Food and Drug Laboratories us it to evaluate various physical and chemical methods for determining vi min A (Murray and Campbell, 1953) and to study the effect of antibiot *on vitamin A utilization (Murray and Campbell, 1956)*

Working directions for this vaginal smear bioassay are given in detail Embree *et al (1957)* The steps involved are

Ovariectomize young rats fed a "maintenance" diet supplying 200 un vitamin A per kilogram One week after operation, transfer rats to a vit min A free diet and, taking vaginal smears daily, determine vitamin A d pletion (squamous cells in smears for 3 successive days)

Supplement randomly selected groups of about ten animals each wi three levels of reference standard and three levels of test material U total doses between *25 and 250* units administered by *stomach tube* tw daily on two successive days Dilute supplements with vitamin A free o so that the same volume of oil is administered to each animal Keep th dose levels in the proportion 1 2 4 Give an equal volume of the diluen oil to a negative control group

Measure the response (the change in vaginal smear picture from squa mous cells to predominately leucocytes) as the number of days from star of dosing until vaginal *smear shows return to original* squamous type cell indicating redepletion (Return depleted rats to the maintenance diet unti needed for another bioassay They can be re used *three or four times* with out loss of sensitivity to vitamin A administration)

Calculate the potency of the unknown test material relative to the ref

erence standard The dose response curve is a straight line function between logarithm of dose and response in days The assay slope is

$$b = \frac{1}{0\,3010} \frac{T_3 - T_1 + T_{3u} - T_{1u}}{N_1 - N_3 + N_{1u} + N_{3u}}$$

where T_1 , T , and T_3 = total response in days for each reference standard and N_1 , N_2 , and N_3 = number of animals in each reference standard group, T_{1u} etc , and N_{1u} , etc , represent response and numbers of animals in the groups supplemented with the unknown material

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tion syndromes Patients with celiac disease, cystic fibrosis of the pancreas sprue, and biliary atresia all absorb fat and vitamin A with difficulty However, those who have pancreatic involvement with a deficiency of pancreatic lipase absorb vitamin A alcohol (or vitamin A aldehyde) much better than they absorb vitamin A esters (Katsampes *et al*, 1953) Consequently a comparison of vitamin A absorption curves showing differences in response to esterified and unesterified vitamin A serves as a differential diagnostic test (Clausen, 1942–43)

(*2*) *Liver storage* The proportion of orally ingested vitamin A deposited in the liver of normal undepleted animals is constant over a wide dosage range (500–10,000 I U) A bioassay based on liver storage response is described by Ames and Harris (1956) (Fig 1) It incorporates as many as possible of the worth while suggestions contributed by innumerable investigators through the years (Foy and Morgareidge, 1948, Guggenheim and Koch, 1944) It also includes a five point common zero design and slope ratio statistical analysis The bioassay is rapid and simple to perform, it has the best precision of any vitamin A bioassay, it can be applied using the same statistical design and analysis to chicks and to other animals from which the liver can be removed for vitamin A analysis at the end of the experiment The steps in the liver storage procedure follow

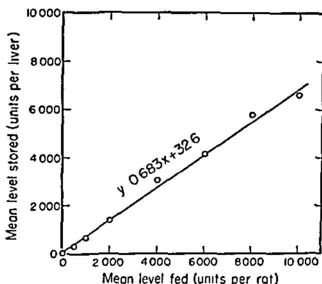


FIG 1 Dose response curve for oral supplement liver storage vitamin A bioassay (*Ames and Harris 1956*)

Feed weanling rats a vitamin A free diet for 9–20 days The animals will have little if any vitamin A in their livers but will not be sick or show any deficiency symptom

Select randomly five groups and supplement the animals with reference standard or unknown test material at levels of 1000 and 2000 units Give

orally (by calibrated dropper to rats and by capsule to chicks) one third of this total dose on each of 3 successive days Do not supplement the fifth group, which serves as a negative control Two days after the last dose, determine the vitamin A in the total liver of each animal by a sensitive procedure such as that described by Ames *et al* (1954) Calculate the relative potency (RP) of the unknown as the ratio of dose response slopes

$$\mathrm{RP} = \frac{b_u}{b_r}$$

where

$$b = \frac{\bar{y}_2 - \bar{y}_0}{2} + \frac{6L_u - L}{70}$$

and

$$b_u = \frac{\bar{y}_{2u} - \bar{y}_0}{2} + \frac{6L - L_u}{70}$$

where

$$L_r = \bar{y}_0 + \bar{y}_{2r} - 2\bar{y}_1$$

and

$$L_u = \bar{y}_0 + \bar{y}_{2u} - 2\bar{y}_1$$

where y_{1r} $\bar{y}_{2r}$, and y_{1u}, $\bar{y}_2$ are the mean values for liver vitamin A in each experimental group y_0 is the mean value for the negative control group

The SE_{RP} (standard error of the relative potency) can be calculated (Embree *et al*, 1957), for this slope ratio, liver storage type bioassay it is ±4% (approximately 95% confidence limits)

The short term, oral supplementation type of liver storage bioassay using chicks gives a measure of the availability of the vitamin A administered Often however, a measure of practical availability is desired, in which availability is modified by the instability of vitamin A in the feed Various investigators have developed such a bioassay in which relatively low levels of vitamin A (in the practical feeding range of 200–5000 I U per pound) are given over relatively long periods of time (3–10 weeks) and the livers are analyzed for vitamin A content (Harms *et al*, 1955 Ely 1959, Olsen *et al*, 1959, de Man *et al*, 1958) The dose response relationship is best evaluated by plotting dose against log liver storage (Fig 2) and calculating relative potency by the statistical procedure reported by Ames (1960) Wilkening *et al* (1960) used the long term, diet supplementation procedure to compare various dry vitamin A preparations and reported good results with levels in the test diet ranging from 200 to 2000 I U per pound

"degrees of protection" (DP of 0 = maximal degeneration and 5 = normal, complete protection) The dose response curve is a straight line relationship between log dose and DP Statistical treatment is the same as for the growth type bioassay (described below in detail), and the precision obtained is approximately ±40%, not as good as that for the growth method

The central nervous system degeneration procedure specifies use of rats placed on a vitamin A free diet 5 days before weaning at 22 days of age Only one sex is used in an assay, otherwise a correction factor must be

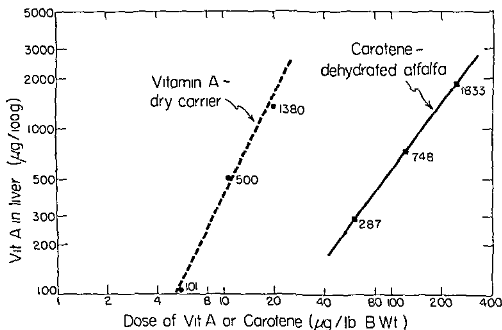


FIG 3 Dose response curve for liver storage vitamin A bioassay using calves (Rousseau *et al* 1956)

employed to adjust for the greater response of the female—about 50% greater than that of the male Comparable groups of ten rats each are started at weaning and fed the vitamin A free diet for 5–6 weeks Both reference standard and test materials of unknown potency are administered orally by calibrated dropper every 3 days in doses of 10, 20 and 40 I U per 3 days The animals are killed, and the brain and spinal cord are removed carefully to prevent injuring the medulla The central nervous system is fixed, stained by the Marchi technique (osmic acid and potassium dichromate), and sectioned for microscopic evaluation The degree of protection is determined for each, and the group response is the mean of the individual DP values

2 Based on Nonspecific Responses

a Growth Young growing animals given a diet containing adequate amounts of every needed nutrient except vitamin A soon stop gaining weight, sicken with intercurrent infection, and die Weanling rats, for example, if their body storage of vitamin A is not too great, will plateau in weight in 3–6 weeks on a vitamin A free depletion diet and will die within 2 weeks thereafter Vitamin A administered to the animals after weight gain has

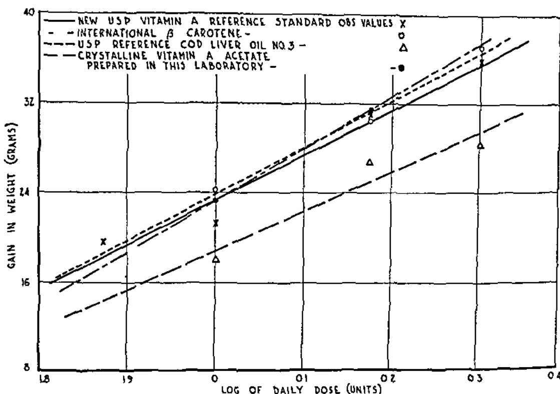


Fig 4 Dose response curves for oral supplement growth vitamin A bioassay (Ellenberger *et al* 1949)

ceased induces resumption of growth at rates proportional to the logarithm of the dose (Fig 4)

Growth bioassays are of two types The curative type is based on a dose response curve relating mean weight gain of groups of depleted animals during a repletion period of 3 or 4 weeks Comparable groups of depleted rats are given graded levels of the reference standard and the unknown test material The critical dose range is 0 5–4 0 I U per day The animals may be given their vitamin A supplement daily or semiweekly

The prophylactic type bioassay is based on the weight gain of groups of undepleted animals fed graded levels of reference standard and test material The test is continued until the negative control animals have died

and the weight gains of the other groups show significant differences Mean weekly body weight gains during the test period are plotted against logarithm of dose, and the straight line dose responses are compared statistically

The prophylactic technique requires more time to run than the curative However, it makes use of healthy, undepleted animals in contrast to the sick, depleted animals used in the curative bioassay One of the most difficult tasks in all bioassay practice is to judge the proper time of depletion The animals must not be too depleted, otherwise the response to vitamin A supplementation will be unpredictable, some will die, and the weight gain of the others will be erratic The bioassay precision will be lowered The animals must not be too undepleted however, or the growth response to vitamin A supplementation will be greater than that due solely to the vitamin A in the supplement being due in part to the residual vitamin A in the tissues of the incompletely depleted animals Both the accuracy and precision of the bioassay will be adversely affected Obviously the judging of properly depleted animals and the making up of comparable groups for supplementation are critically important steps in the curative procedure Richards and Simpson (1934) carefully studied and experimented with many variables in the curative method, attempting to improve reproducibility of results They concluded that formulation of a satisfactory bioassay of the curative type was hopeless Many others concur

Making up comparable groups of animals is also difficult in the prophylactic type bioassay since the animals have significant but variable stores of vitamin A Thus the growth response to vitamin A supplementation will be variable Investigators attempting to apply the prophylactic technique using chicks find excessive variability in response similar to that experienced by investigators using rats A typical example was a test on a series of commercial dry vitamin A preparations, using growth and liver storage in chicks as criteria of response (Olsen *et al* 1959) The experiments were well planned and carefully conducted Four replications with large numbers of chicks were used, and management of the animals was excellent The newly hatched chicks used were from flocks on restricted vitamin A intake and had very little vitamin A in their bodies at hatching No attempt was made to deplete them The birds were fed the supplemental diets for 4–7 weeks at levels ranging from 100 to 1200 I U per pound The growth response was so variable that the authors concluded that short term prophylactic growth type bioassays are not reliable for evaluating vitamin A activity

Other investigators (de Man *et al* 1958, Wilkening *et al*, 1960) find using partially depleted birds, that growth response over 4 to 9 week periods is a satisfactory criterion The problem of determining the proper stage

of depletion at which to start the curative growth bioassay in chicks is as critical and difficult as it is in rats

Theoretically the prophylactic technique is better than the curative, but practically the curative type bioassay is the method of choice and has been the most widely studied and used of any bioassay Since 1948, when a physicochemical procedure replaced it as the official method (U S P XIV) for determining vitamin A, the rat growth bioassay has been used less frequently, but it is still preferred by some investigators

Working directions for the rat growth bioassay are given in detail in Embree and co workers' review chapter (1957) The steps in the bioassay are

Feed weanling rats a vitamin A free diet until they are depleted Use either only male or only female rats with low vitamin A reserves A rat is *considered depleted when all the following conditions are met (1) a net* weight gain in 4 days of 1 gm or less, (2) failure to gain on 2 of the 4 days, (3) length of time on the depletion diet not less than 18 or more than 45 *days*

Assign depleted rats randomly to groups of at least ten and weigh and supplement twice a week for 4 weeks Administer the reference standard to *two groups at levels of 6 and 12 units per week and give the unknown test* material at the same levels Keep one group as a negative control

Calculate the mean weekly gain T for each group and determine the assay slope

$$b = \frac{1}{0.3010} \frac{T_2 - T_{1r} + T_{2u} - T_{1u}}{0.5(N_r + N_u)}$$

where $T_1 + T_{2r} = T$ (mean weekly gain for groups fed reference standard) *and* $T_{1u} + T_{2u} = T_u$ *(mean weekly gain for groups fed unknown* test material) and N_r and N_u = *total number of animals in reference and* in unknown test material groups, respectively

Calculate the relative potency of the unknown

$$\mathrm{RP} = \mathrm{antilog}\,\frac{1}{b}\left(\frac{T_u}{N_u} - \frac{T_r}{N}\right)$$

The $\mathrm{SE_{RP}}$ *(standard error of relative potency)* can be calculated (Embree *et al*, 1957) For this growth type bioassay the $\mathrm{SE_{RP}}$ is approximately ±25%

b Survival A single dose of test material is given to vitamin A depleted rats The response, survival time of the animal, is easily and accurately determined and is proportional to the vitamin A content of the material fed By measuring the area under the curve relating weight to survival days a good measure of vitamin A activity is obtained (Sherman and Todhunter, 1934)

Ellingson and co workers (1951) in a study comparing the potency of three vitamin A products for pediatric use found comparable results using growth, liver storage, and survival bioassays. Their technique for the survival bioassay was to use vitamin A depleted rats, administer a single dose of vitamin A by stomach tube, and determine the number of days until each rat died. The doses of vitamin A were 14, 28, 56, 84, and 140 units in a volume of 0.4 ml of diluent. The responses were directly related to the log dose and ranged from about 20 days for the lowest dose to about 60 days for the highest.

This method should be useful for the analysis of materials administered topically or parenterally. Slow release preparations might be more accurately bioassayed by the single dose survival technique than, for example, by plasma vitamin A concentrations. Not enough work has been done to establish the precision of the survival bioassay.

3 Based on Special and Miscellaneous Responses

a Hypervitaminosis A. Rats fed massive doses of vitamin A lose weight, develop fragile bones, and undergo adrenal gland enlargement. These criteria of response are easily and precisely measured and are proportional to the logarithm of dose (Ames *et al.*, 1952, 1954). However, should the hypervitaminosis A test be considered a bioassay of vitamin A activity—a bioassay at the high end of the dose spectrum as the growth and vaginal cornification techniques are at the low end, and the liver storage method at the middle portion of the dose spectrum?

One objection to its use is the large amount of test material and reference standard needed to supply the massive doses (about 100 000 I U per kilogram body weight per day) for groups of animals over a 3–4 week experiment.

A more serious drawback is the difficulty of interpreting the response if it differs significantly from that of vitamin A. An example is the bioassay of vitamin A acid, which Arens and van Dorp (1946a) reported as being 10% as potent as vitamin A by growth bioassay. The sodium salt is 100%. Conversely, by liver storage bioassay vitamin A acid has zero potency because it is not deposited in the liver as vitamin A even after administration of large doses (Arens and van Dorp, 1946b, Sharman, 1949). Using another criterion of response Hohlweg (1948) reported that for protecting the germinal epithelium of vitamin A deficient male rats against degeneration vitamin A acid is 100 times more active than vitamin A as fish liver oil. What response does vitamin A acid give in the hypervitaminosis A test?

Ames and co workers (unpublished data) compared vitamin A acetate, vitamin A aldehyde, and vitamin A acid (all crystalline all *trans* compounds) by feeding massive doses to young rats. Their results, shown in Table II, indicate that vitamin A acid induces the same growth inhibition

the same decrease in bone breaking strength, and the same adrenal hypertrophy as does vitamin A acetate—but at doses one tenth as high. Should these results be interpreted to mean that vitamin A acid is ten times more active (potent) than vitamin A acetate? Or that this is *not a valid bioassay* procedure, but merely a toxicity test showing that vitamin A acid is about ten times more toxic than vitamin A acetate?

TABLE II

HYPERVITAMINOSIS A TEST

Responses	Dose (mg./100 gm. body weight)			
	1.8	3.6	5.4	10.8
Mortality (%)				
A acetate	0	0	0	20
A aldehyde	0	20	40	100
A acid	0	60	100	100
Weight gain (gm./week)				
A acetate	30	28	28	19
A aldehyde	28	24	16	—
A acid	13	7	—	—
Adrenals (mg./100 gm. body weight)				
A acetate	24	25	22	49
A aldehyde	23	39	47	90
A acid	45	84	110	—
Bone strength (% of control)				
A acetate	79	69	56	33
A aldehyde	70	52	33	30
A acid	35	33	—	—
Liver vitamin A (I.U./liver)				
A acetate	73,000	107,000	103,000	103,000
A aldehyde	50,000	75,000	85,000	60,000
A acid	0	0	0	0
Liver storage efficiency (%)				
A acetate	58	44	29	21
A aldehyde	43	33	29	15
A acid	—	—	—	—

Lewis and Cohlan (1952), using hypervitaminosis A as the approach, found vitamin A in aqueous dispersion significantly more active (toxic) than vitamin A in oil.

b. In vitro Opsin Assay for cis Isomers. The problem of determining the individual isomers of vitamin A in a mixture of isomers is both formidable and important. Most of the *cis* isomers are relatively inactive biologically compared with the all *trans* form. Although so called neovitamin A (2 *cis*) is about 75% as potent as all *trans* vitamin A, 6 *cis*, 4 *cis*, and 2,6 di *cis*

are only 20–25% as potent. Subjects of very active current research include the natural occurrence of the various isomers, their interconversion *in vivo*, and the artificial production of *cis* isomers from all *trans* vitamin A during the processing of fish liver oil and the storage of certain pharmaceutical preparations (aqueous multivitamin dispersions).

Infrared analysis of isomer mixtures is possible, but concentration and purity of the mixtures must be high. This is a research procedure that is not amenable to routine use on low potency extracts.

A chemical method based on the reaction of maleic anhydride with those isomers having *trans* conformation at both the 2,3 and 4,5 double bonds gives the maleic value (MV) of the isomers. The 2 *cis*, 4 *cis*, and 2,6 di *cis* isomers do not react, consequently they have high maleic values. Ames and Lehman (1960) describe the maleic value determination and its interpretation. They compare maleic values with chick oral supplementation and liver storage bioassay results of fish liver oils and other animal feed sources of vitamin A and find a relationship which can be expressed by the cubic regression

$$\text{relative biopotency} = 99.5 - 0.2\,(MV) - 0.051\,(MV)^2 + 0.000768\,(MV)^3$$

This same relationship between biopotency and maleic value was reported for various isomer mixtures resulting from purposeful isomerization of all *trans* vitamin A palmitate (Ames *et al.* 1960).

A more specific analytical method was proposed by Hubbard and associates (1953), based on the *in vitro* reaction of opsin with certain vitamin A aldehydes to form visual purple which can be measured spectrophotometrically. Plack (1959) developed the opsin technique and applied it to the determination of 4 *cis* isomer content of vitamin A extracted from crustaceans and from rat livers. Brown *et al.* (1959) also employed the opsin assay to analyze various isomer mixtures for 6 *cis* isomers. The results corresponded very well with infrared analysis. Plack assumed that 6 *cis* isomers were absent from his extracts and determined the amount of rhodopsin, λ_{max} 500 mμ, formed as a measure of 4 *cis* vitamin A. Brown *et al.* assumed that 4 *cis* isomers were absent from their extracts and determined the amount of isorhodopsin, λ_{max} 487 mμ, formed at two different time intervals as a measure of 6 *cis* + 2,6 di *cis* isomers. The following outline of the Brown *et al.* (1959) procedure for the estimation of 6 *cis* vitamin A isomers exemplifies an opsin bioassay.

The opsin needed is prepared from cattle eyes by standard protein isolation technique and tested for activity (Brown *et al.* 1959, Plack, 1959). (Occasional batches are inactive for no apparent reason, but an active batch once prepared retains its potency indefinitely if kept at −15°.)

The unknown to be assayed may be vitamin A extracted from a pharmaceutical product, from a synthetic organic chemical reaction mixture, from

liver tissue, etc , but under the conditions of this test only two isomers, the 6 *cis* isomer (iso a) and the 2,6 di *cis* isomer (iso b) react with opsin to form isorhodopsin, λ_{max} at 487 mμ The 4 *cis* isomer, neo b, which would react with opsin to form rhodopsin, λ_{max} at 500 mμ, is presumed to be absent (present only in retinal tissues) Also the all *trans* and 2 *cis* (neo a) isomers, if present, do not react with opsin Prior to the testing in the opsin system, the vitamin A isomers in the unknown mixture are oxidized to the respective retinenes by passing through a small column of manganese dioxide (Henbest *et al* , 1957)

Mix the opsin (in excess) and unknown mixture of retinenes in each of two microcells of a spectrophotometer and incubate at 20–25° for 2 hours At this time all the 6 *cis* retinene (iso a) has reacted with opsin, and 21% of the 2,6 di *cis* retinene (iso b) is isomerized and has reacted with opsin to form isorhodopsin The reaction is stopped in the first cell by adding hydroxylamine to trap unreacted retinenes, the absorbance at 487 mμ is measured, the mixture is bleached, and the extinction (E) at 487 mμ re measured The difference in E values is a measure of the rhodopsin formed by 100% of the 6 *cis* and 21% of the 2,6 di *cis* retinenes present in the sample After 24 hours the reaction is stopped in cell 2 and the difference in E values at 487 mμ determined as before This difference is a measure of the rhodopsin formed by 100% of the 6 *cis* and 100% of the 2,6 di *cis* retinenes in the sample The proportion of these two isomers to the amount of total retinenes can then be calculated

Using this technique, Brown and co workers (1959) found 19–26% 6 *cis* isomers (combined amount of 6 *cis* and 2,6 di *cis* vitamin A) in some fish oils, 14% in a rat liver oil, and 20–23% in equilibrated retinene isomer concentrates (Table III)

c Kidney Function The kidney undergoes definite pathological changes in vitamin A deficiency that are reflected in abnormal kidney function In the chick, massive deposits of urates are found around the heart, liver, and kidneys, the condition is diagnosed as visceral gout Elvehjem and Neu (1932) found that the blood uric acid of vitamin A deficient chicks was increased eight to ninefold over a normal value of about 5 mg /100 ml

In dogs, Herrin and Nicholes (1939) found that vitamin A plays an im portant role in renal physiology Results of urea clearance and inulin clearance tests in young vitamin A deficient dogs were significantly de creased Administration of either vitamin A or carotene returned the values to normal

The adequacy of kidney function tests as the criterion of response for a vitamin A bioassay would be interesting to investigate further

d Other A provocative paper by Lowe and co workers (1953) showed that in the terminal stages of vitamin A deficiency previously unknown

metabolites appeared in the unsaponifiable portion of the liver, also that histochemical changes occurred in the adrenals and that degeneration took place in epithelial tissues in the region of xerophthalmic lesions These newly established specific symptoms of vitamin A deficiency may be used as criteria of response if subsequent investigation confirms their physiological significance and if they begin to develop early in the deficiency Some of these constituents of the unsaponifiable fraction of the liver that change

TABLE III

OPSIN ASSAY FOR 6 *cis* ISOMERS OF VITAMIN A

Sample No	Description	Percentage *iso* a + *iso* b in total vitamin A isomers	
		Opsin assay	Infrared assay
1	Shark nonsaponifiable $E^{1\%}_{1\,m}$ (328 mμ) = 538	24	—
2	Shark distilled nonsaponifiable $E^{1\%}_{1cm}$ (328 mμ) = 628	26	—
3	Cod nonsaponifiable $E^{1\%}_{1\,m}$ (328 mμ) = 202	19	—
4	Distilled cod nonsaponifiable $E^{1\%}_{1\,m}$ (373 mμ) = 427	25	—
5	Mixed fish liver oil nonsaponifiable $E^{1\%}_{1\,m}$ (373 mμ) = 350	19	—
6	Rat liver oil nonsaponifiable $E^{1\%}_{1\,m}$ (370 mμ) = 270	14	—
	Equilibrated retinene isomer concentrates		
7	$E^{1\%}_{1\,m}$ (372 mμ) = 1030	21	18
8	$E^{1\%}_{1\,m}$ (377 mμ) = 1260	23	22
9	$E^{1\%}_{1\,m}$ (374 mμ) = 1080	20	21

Brown *et al* (1959)

with the vitamin A status of the animal have already been described (constituent SC λ_{max} 230 mμ and hydrocarbon, λ_{max} 260 mμ) and identified (ubiquinone 50), but their exact relationship to vitamin A metabolism is still being sought (Morton and Phillips, 1959)

A water soluble metabolite of vitamin A in urine, discovered by Wolf *et al* (1955, 1957) during studies with C^{14} labeled vitamin A is very interesting It is a ketoester accounting for 5–12 % of the dose of vitamin A and may be suitable as the basis of a bioassay if it is easy to measure and is shown to be related to dose of vitamin A administered

Two groups, Patterson *et al* in Canada (1942) and Mayer and Krehl at Yale University (1948) independently found that efficiency of food utiliza

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The Conversion of β-Carotene into Vitamin A

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I Introduction

The relationship between the vitamin A activity of the yellow carotenes of plants and the colorless growth factor in liver oils became clearer after Moore (1930) demonstrated that "carotene" was metabolized in the animal body to form vitamin A, which was stored in the liver The resemblance of the structure of vitamin A, elucidated by Karrer *et al* (1931), to half the β carotene molecule suggested to them that it might be formed from the latter by central fission involving the addition of the equivalent of two molecules of water The enzyme "carotenase" concerned was then sought in liver tissue where the vitamin appears in highest concentration The experimental evidence at that time for its presence in liver yielded conflicting results, and in general the case was regarded as not proved (Woolf and Moore, 1932)

Although everyone agreed that β carotene administered orally was transformed into vitamin A great difficulty was encountered in demonstrating the conversion when the provitamin was administered parenterally (Rokhlina *et al*, 1943, Sexton *et al* 1946) This early work has been reviewed briefly by Glover *et al*, (1948b) When a positive conversion was observed after the injection of β carotene, the amount of vitamin A formed was invariably small and little or no storage took place although the β carotene reached the liver in measurable amounts Good dispersion of the provitamin

with a surface active agent appeared essential (Tomarelli *et al*, 1946) The more recent experiments of Bieri and Sandman (1951) on rats also indicate that good dispersion is necessary, but for good growth five times as much β carotene is required intramuscularly as orally

The more efficient conversion by the oral route eventually led a number of workers independently to the establishment of the small intestine as the main site of conversion in a number of animal species (Mattson *et al*, 1947, Glover *et al*, 1947, 1948b, Thompson *et al*, 1947, Goodwin and Gregory, 1948) Since then the experiments of Bieri and Pollard (1954), McGillivray and co workers (1956), and Worker (1956, 1959) have demonstrated that vitamin A can be formed from β carotene in other animal tissues, but only to a slight extent compared to the small intestine The greater efficiency of the intestine can perhaps be attributed in some measure to its excellent system for dispersing lipids and making the fat soluble β carotene more accessible to the enzyme system Herbert and Morgan (1953) observed that if β carotene is administered orally dispersed in Tween 40 it is utilized better than if given in oily medium Alternatively, the concentration of the enzyme system may be higher in the small intestine than in other tissues

Attempts have been made to trace the site of formation of the vitamin within the intestine by fluorescence microscopy Popper (1941) and Greenberg (1957) claim to have detected fluorescence attributed to vitamin A, first, within the lumen adjacent to the villi as rapidly as 5–15 minutes after the oral administration of β carotene and later in the mucosa and lamina propria This would suggest that the initial attack on the molecule took place extracellularly

However, chemically, Glover *et al* (1948b) were unable to detect vitamin A in the fluid from the intestinal lumen of rats dosed with β carotene These different findings may arise on account of the difference in sensitivity of the techniques for vitamin A A difficulty with the fluorescence technique is ensuring that the fluorescence observed really arises from the compound under investigation

II Efficiency of the Conversion

If the β carotene molecule could be split in the animal organism at the central double bond, then two molecules of vitamin A would result This "central fission" hypothesis was supported by the early comparative work of Kuhn and co workers (1933) and von Euler *et al*, (1934) on the biological activity of the different carotene isomers They showed that α and γ carotene had only half the biological activity of the β isomer When vitamin A was obtained in a pure crystalline form (Holmes and Corbet, 1937) and its biological potency in the growth test compared with β carotene, it was

unexpectedly found to be twice as active weight for weight Thus only one molecule of vitamin A was formed from one molecule of β carotene Many subsequent biological assays have confirmed this result (Mead *et al*, 1939, Hume, 1951) This raised the possibility, expressed by Morton (1940) in reviewing the situation, that fission occurred asymmetrically For this to fit the evidence satisfactorily the assumption has to be made that the two ends of the conjugated system of double bonds in the various carotenoids are equally susceptible to attack Otherwise, γ carotene with one end apparently more accessible to oxidative enzymes would have a biological activity equivalent to that of β carotene Alternatively, it might be postulated that half the dose of β carotene molecules was split centrally while the other half was completely degraded by a different oxidative enzyme Thus, whereas the maximum efficiency of utilization of β carotene appeared to be close to 50 %, perhaps under ideal conditions it would be possible to attain 100 % conversion Support for both these alternatives was soon provided

1 Terminal Oxidation

On physicochemical grounds Zechmeister and co workers (1943) suggested that because of resonance, the central double bond of a conjugated system will be more stable than a terminal one and therefore less susceptible to attack Certainly the evidence from the products formed by the action of various inorganic oxidants on β carotene confirms this concept Chromic trioxide appears to attack the double bonds of the β ionone rings preferentially, the end products being semi β carotenone and β carotenone (Kuhn and Brockmann, 1935) Alkaline permanganate on the other hand, appears to attack the ends of the connecting chain of conjugated double bonds yielding long chain aldehydes, the β apocarotenals, having one β ionone ring intact (Karrer and Solmssen, 1937) Although retinene can be formed in small yield by the action of this reagent on vitamin A (Morton and Goodwin 1944), it has not been detected among the permanganate oxidation products of β-carotene With hydrogen peroxide, retinene is formed in small yield (Hunter and Williams, 1945), and in the presence of osmium tetroxide as catalyst in good yield (Goss and Macfarlane, 1947 Wendler *et al*, 1950, Glover and Redfearn 1954, Grob and Butler, 1954) Following the progress of the reaction with time Glover and Redfearn (1954) were able to prepare the whole series of β apocarotenals and obtained evidence that indicated that the penultimate bond in the conjugated system was the one preferentially attacked by the H_2O_2 osmium tetroxide reagent

The finding that retinene was readily reduced to vitamin A *in vivo* (Glover *et al*, 1948a) strengthened Hunter's suggestion (1946) that oxidative attack was the mechanism for the conversion process, but the problem remained

at which point along the conjugated double bond system did the initial reaction take place

Further support for the terminal oxidation hypothesis was provided by the fact that β apo 8′ carotenal had been found to be biologically active (von Euler *et al*, 1938) Again, two carotenals were found in the intestine of the horse (Festenstein, 1951), which were later tentatively identified as β apo 10′ carotenal and β apo-12′ carotenal, respectively These were not hitherto known to occur in plant products and consequently were regarded as possibly being formed *in vivo* More recently, they have been detected in certain plants by Winterstein and Isler (private communication) so they might have been partly derived from the food

Again, 16,16′ bishomo β carotene is biologically active, although it does not possess a central double bond and the metabolism of other molecules, such as cryptoxanthin or α carotene, known to form vitamin A has never led to the storage in the rat of the hydroxy- or α "vitamin A" corresponding to the inert halves of the carotenoids Yet α "vitamin A" is readily stored in the animal body (Ames *et al*, 1955)

2 Central Fission

A considerable difficulty with the terminal oxidation hypothesis is that compounds intermediate in size between β carotene and vitamin A have not been detected in experimental animals given large doses of the former, yet spectroscopically they should be detectable in microgram quantities It may be, however, that they are present only in an activated form and therefore water soluble or strongly bound to protein

Again, we cannot predict accurately how a molecule will behave in a biological system from its behavior in pure solution The possibility therefore of an initial attack on the molecule closer to the center cannot be completely excluded

The work of Moore (1940) strengthened the idea that the efficiency of the conversion process might be greater than 50% He noted that the vitamin A requirements of rats were greater in vitamin E deficiency Later it was shown by a number of workers that vitamin E enabled a greater amount of vitamin A to be stored in the liver after a given dose of β carotene

Hickman and co workers (1944) demonstrated that the effect was antioxidative in nature and that an optimal amount of the vitamin E (0 5 mg per day) had to be administered along with the carotenoid This sparing action of vitamin E was more marked with the carotenoids than with vitamin A Larger doses up to 10 mg per day, however impair the utilization of β carotene (Johnson and Baumann, 1948) Similarly, some other artificial antioxidants reduce the yield of vitamin A from a given dose of β carotene while showing no effect on the absorption of vitamin A itself (High *et al*, 1954)

The oxidative destruction observed by Hickman and Moore may be due to the action of a lipoxidase enzyme Hove (1943) demonstrated in the mucosa of rat stomach the presence of such an enzyme which could destroy β carotene in the presence of methyl linoleate The action of this animal lipoxidase may be similar to that of some of the plant oxidases, which are known to degrade β carotene in the presence of linoleate to form long chain aldehydes (Friend, 1960) The latter can be metabolized to vitamin A *in vivo*, but the yield is low (see below)

Biological assays of β carotene in the presence of the optimal amount of vitamin E have been carried out by Koehn (1948), and by Burns *et al* (1951) Both have reported that 1 μg β carotene has a biopotency equal to 1 μg vitamin A, indicating 100% efficiency of conversion, that is, central fission In view, however, of the many other assays that indicated that only one molecule of vitamin A resulted from one molecule of β carotene, and none giving an intermediate value, it would be unwise to draw any definite conclusion from the biological assay regarding the mechanism of the reaction Even at low dose levels, different proportions of the two sub stances may be absorbed or destroyed

3 In Vitro *Experiments*

A major difficulty in examining the problem of the mechanism of the conversion process is that it occurs only on a very small scale in experi mental animals, and relatively slowly Many attempts have been made to prepare an enzyme that will carry out the reaction on a reasonable scale *in vitro* The results so far have been mainly negative (Glover *et al*, 1948b, Kon and Thompson, 1951, Bieri and Pollard 1953) or on a minute scale (Rosenberg and Sobel, 1953, Olson, 1959) Recently, thorough tests of the capacity of various isolated organs and homogenates to carry out the re action have been conducted by Worker (1959), who has had limited suc cess Further tests with improved techniques were carried out recently in our laboratory (Shah, 1958) with everted intestinal sacs of the rat, again without observed formation of vitamin A The β carotene was dispersed in a glucose medium buffered with phosphate and containing bovine al bumin (5 mg /ml) as dispersing agent Incubation was carried out for 2 hours at 37°C in a 95% O_2/5% CO_2 atmosphere During the experiment the color of the medium changed from red to pale brown as the β carotene became complexed with the mucoproteins and better dispersed In this sys tem which has been used successfully in the study of other lipids, the β carotene can be taken up by the mucosal cells At the end of the experi ment the mucosal cells were scraped off and extracted with ether, along with the medium, after the proteins were denatured with ethanol The β carotene was recovered virtually quantitatively but no vitamin A was found

Similar experiments were also carried out with echinenone (4′ keto β carotene), which already has an oxygen function at one end and is known to be a provitamin (Lederer and Moore, 1936, Shah, 1958), and again no vitamin A was formed

4 In Vivo *Experiments*

Recently, a claim has been made that retinene is present as a protein complex in the intestine of rats during the absorption of β carotene, but the amount isolated by paper chromatography was not really sufficient for proper identification (Suzuki *et al*, 1959) The finding of a bound form of retinene in herring roe and eggs by Plack, Kon, and Thompson (1959) is of considerable interest Whether these complexes are formed from β carotene directly or from vitamin A will have to await further experiments on their chemistry This knowledge will be of considerable value in planning future experimental work All the disappointing results with live animals and isolated tissues in the study of this conversion problem prompted an alternative approach

III Metabolism of Hypothetical Intermediates

The principle of this procedure is to study the metabolism of synthetic compounds intermediate in size between β carotene and vitamin A which could possibly be closely related to the true biological intermediates Since analogies have often been drawn between enzymatic oxidations and those brought about by hydrogen peroxide (Dakin, 1922), it seemed worth while to study the metabolism of the products of the oxidation of β carotene by this reagent Hypothetical schemes such as that presented in Fig 1 may be followed The β carotene is visualized as undergoing a form of β oxidation at the activated aldehyde or acid level to form the intermediates listed (Fig 1) The concept of terminal oxidation can be examined in greater detail this way Support for the hypothesis that the initial attack may take place at a terminal double bond was provided by the finding that β apo 8′ carotenal is biologically active in the rat growth test (von Euler *et al*, 1938), and this compound formed a good starting point

If it resulted from the oxidation of a terminal double bond in the chain then it or the corresponding acid may be degraded further by a β oxidation type enzyme system The 9′ and 13′ branch methyl groups lie in α position to potential carboxyl groups and would not stop β oxidation but only inhibit it However, when the central carbon atom is reached, β oxidation would be blocked by the methyl group attached to C 13 which is in the β position to the central carbon atom of β carotene

It was found possible to isolate chromatographically several of these β apocarotenals in a fairly pure state from among the products of the oxi

dation of β carotene with the Milas Sussmann reagent (Wendler *et al*, 1950, Glover and Redfearn, 1954) Most of them have now been synthesized in the pure all *trans* form by Ruegg and co workers (1959a,b) It was hoped that by examining the behavior of these substances after administration to rats, some information would be obtained regarding the enzymatic attack on the molecules and the manner in which they react *in vivo*

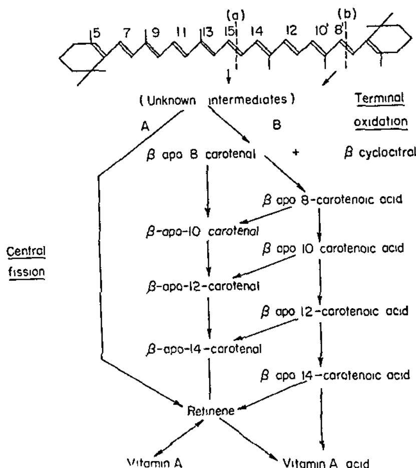


FIG 1 Possible pathways of β carotene metabolism

Several of the β apocarotenals have been administered orally to vitamin A deficient rats, and the amount of vitamin A formed in the intestine and stored in the liver was estimated and compared with the usual amount obtained from similar doses of β carotene The results are given in Table I The degree of conversion of each to vitamin A was disappointingly low, less than 4%

These values compared with a minimum yield of 10% from β carotene at a comparable dose level do not fit in easily with what might be expected

of intermediates in the conversion process It was felt, however, that vitamin A formation was a better criterion of function as an intermediate than growth because the latter can be maintained by vitamin A acid, which could be formed by a secondary route rather than through vitamin A On the other hand, all were definitely converted to vitamin A as characterized by accepted spectrophotometric and color tests, so if some β carotene is oxi-

TABLE I

PROVITAMIN A ACTIVITY OF SOME β APOCAROTENOIDS IN THE RAT

β Apocarotenoid	*Biological activity*		
	Daily *requirement* (μg)	Vitamin A formation	
		Dose (mg /rat)	Vitamin A (%)
C_{20}			
Vitamin A	1	15	40–70
Retinene	1	1 7–8 0	50–70
Vitamin A acid	12	10	None
C_{22}			
β Apo 14 carotenol	30	—	—
β Apo 14 carotenoic acid ester	50–100	2 5	4
2 (15 Hydroxyretinyl)acetic acid ester	5–10	2 5	3
C_{25}			
β Apo 12′ carotenal	2	0 9	4
β Apo 12 carotenoic acid ester	1	1 5	0 5 (24 hr)
		0 5	7 0 (60 hr)
γ (15 Hydroxyretinyl)tiglic acid ester	<2	2 5	21
C_{27}			
β Apo 10 carotenal	—	1 5	Trace
C_{30}			
β Apo 8 carotenal	~5	0 6	3
β Carotene	2	1–4	10–15

dized at an eccentric position to form β apocarotenals, then these can be metabolized in turn to form vitamin A Plant lipoxidases are known to form β apocarotenals from β carotene (Friend, 1960) It may be that they can also be formed in the animal If so, it may be claimed that they are intermediates in a nonspecific route from β carotene to vitamin

One could perhaps take the alternative view that the poor yield of vitamin A is due either to the fact that the real intermediates are in an activated form which cannot easily interchange with these synthetic compounds *or* to overloading of the conversion system, certainly the growth promoting

activity done at low dose levels showed the C_{25} compounds to be as good as or better than β carotene Quantitative bioassays of the series of β apocarotenals from C_{25} to C_3 by Marusich *et al* (1959) have confirmed the above observations that the C_{25} compounds are more active than all *trans* β carotene in the growth test β Apo 12′ carotenal has 125 % (range 86–182) activity versus all *trans* β carotene taken as 100 %

It was observed during these studies that the aldehydes were readily oxidized to the carboxylic acids, so presumably retinene also can be metabolized this way to vitamin A acid, which has proved difficult to detect after presentation to animal tissues These unsaturated carboxylic acids can be esterified, but most are usually in the free form They are also partly reduced *in vivo* to the corresponding carotenols but not quite so readily as the shorter chain compound, retinene

If normal β oxidation were involved then the β apocarotenoic acids might be closer to the true intermediates, so two of the series, the C_{22} and C_{25} compounds were synthesized (Fazakerly and Glover, 1957) and their metabolism studied At the same time two hydroxy acid esters 2 (15 hydroxyretinyl)acetic acid ester and γ (15 hydroxyretinyl)tiglic acid ester intermediates in the synthesis were also examined for biological activity The summarized results are included in Table I Again vitamin A was formed in varying yields from each of the compounds administered The C_{25} hydroxy acid was the only compound which gave a better yield of vitamin A than β carotene The yield from β apo 12′ carotenoic acid was much lower, the vitamin being formed only very slowly In the test of minimal requirement for steady growth, however, the C_{25} aldehyde and acid were as good as vitamin A itself whereas the C_2 alcohol and acid were very inferior yet the latter are closer structurally to vitamin A

Since the C_2 compounds would be intermediate in the metabolism of the C_{25} series by β oxidation, it is quite clear that this enzyme is not operative in the metabolism of the C_{25} acid and presumably also of the C_{25} aldehyde It is of some interest that the C acid can be metabolized to the lower alcohol vitamin A, whereas vitamin A acid was not detected It is well known that vitamin A acid cannot be reduced to the alcohol, so perhaps during the metabolism of the C_2 compound an intermediate active "aldehyde" can be formed which can be acted on by alcoholic dehydrogenase to release vitamin A The process may be similar to that involved in the formation of aliphatic aldehydes and alcohols from the higher acids

The general conclusion from the above experiments is that although terminal oxidation of β carotene can yield vitamin A, β oxidation of the larger fragment is not the method by which it is degraded to vitamin A

It was noted that the C_{25} ester isolated from the tissue had an absorption maximum about 5 mμ higher than the material administered The

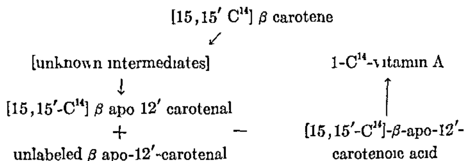
change probably arises through the gradual transformation of some *cis* form to the more stable all *trans* form during hydrolysis and re esterification as the substance was being absorbed In the intestine about 40% of the material was in the *trans* form whereas in the liver it had risen to 90%, approximately The esters are absorbed better than the free acids

Of all the compounds examined, only the C_{25} series looked like being closely related to intermediates in the metabolism of β carotene In order to test whether in fact they were intermediates, a more critical experiment was attempted (Glover and Shah, 1958) with β apo-12′-carotenal and specifically labeled [15,15′ C^{14}]β carotene* (Inhoffen *et al*, 1955)

1 *Metabolism of [15,15′ C^{14}] β carotene*

β Apo 12′ carotenal was selected as representative of the intermediates because it can be readily oxidized to the corresponding acid and if activation is necessary could probably enter the enzyme system more easily The Scheme 1 illustrates the aim of the experiment, which was to use the C_{25}

SCHEME 1 OXIDATION OF β CAROTENE



aldehyde or the acid formed from it as trapping agents for any radioactive C_{25} aldehyde or acid which *would be formed from the [15,15′ C^{14}] β carotene* during its metabolism to vitamin A Two experiments were carried out, using *two vitamin A deficient rats in each* The animals were dosed with a mixture of the labeled β carotene and β apo 12′ carotenal in slight excess dissolved in about 1 0 ml arachis oil, which incidentally contains about the optimal amount of vitamin E for the conversion process The animals were then killed 5 hours after dosing, when absoprtion across the intestine is generally optimal The lipids were isolated from the intestine and liver and resolved chromatographically The β 12′ apocarotenal was isolated as the crystalline oxime The acid fraction was too small to crystallize as a derivative, so it was purified by quantitative conversion to the ester, chromatographed, and then reduced to the corresponding alcohol and rechromatographed

* The author wishes to thank Professor G Mackinney for the gift of this material

The specific activities are given in Table II, alongside those for the original C^{14} labeled β carotene and the C^{14} vitamin A isolated from the livers The small amount of radioactivity in all the C_{25} compounds is indicative that a little β carotene was degraded to β apo 12′ carotenal and the fact that aldehyde oxidase action is irreversible implies that the aldehyde became labeled first Again, the agreement in the specific activities of the aldehyde and the acid indicates that none of the latter can have been formed via an alternative route, e g from a higher homolog by β oxidation The aldehyde reisolated from the lumen was radioactive, so the [15 15′ C^{14}] β apo 12′ carotenal must have been formed there If the simple assumption is made that the whole aldehyde is used to dilute the C^{14} labeled

TABLE II

METABOLISM OF [15 15 C^{14}] β CAROTENE IN THE PRESENCE OF β APO 12 CAROTENAL IN THE RAT

Compound	Specific activity (c /m /μmole)			
	Dose	I	Dose	II
[15 15 C^{14}] β Carotene	4 5 mg	197 000	2 39 mg	361 000
Unlabeled β Apo 12 carotenal	7 9 mg		5 3 mg	
[15 15 C^{14}] β Apo 12 Carotenal oxime		641		2,520
[15 15 C^{14}] β Apo 12′ carotenoic acid ester		615		—
[15 15 C^{14}] β Apo 12 carotenol		605		—
[15 C^{14}] Vitamin A ester		1 326		3 890
[15 C^{14}] Vitamin A alcohol		1 490		3,400

β apo 12′ carotenal, then about 1–3% of the β carotene can have been metabolized via that compound The specific activity of the labeled vitamin A from the liver was higher than that expected from the labeling in the carotenoic acid, showing that it was probably formed mainly by another route

2 Metabolism of [U C^{14}] β Carotene and [U C^{14}] Retinene

The metabolism of [U C^{14}] β carotene and [U C^{14}] retinene has been examined with a view to determining how extensively they are degraded during absorption across the intestinal tract Single doses of retinene were known to be absorbed from the intestine in good yield (Glover *et al* , 1948a), so the labeled material was not therefore expected to be metabolized into fragments that would eventually release $C^{14}O_2$ into the respired air as quickly or as extensively as the [U C^{14}] β carotene, if the latter is oxidized and degraded from one end On the other hand, if central fission is the main

route for the metabolism of β carotene, the pattern of release of $C^{14}O$ in the respired air would tend to be similar to that for the C^{14} retinene

[U C^{14}] β Carotene has been synthesized from $C^{14}O_2$ by photosynthesis in barley seedlings (Willmer and Laughland, 1957) and in the blue green alga *Anabaena variabilis* (Glover and Shah, 1957) and its metabolism studied in rats (Willmer and Laughland, 1957, Fishwick and Glover, 1957) Both groups of workers found that it was extensively degraded to small fragments since $C^{14}O_2$ appeared in the respired air within half an hour after dosing and attained its maximum value 4–5 hours after dosing, when absorption across the intestine is optimal, and then it declined Approximately 2–3 % of the β carotene was metabolized to $C^{14}O$ over the 24 hour period The sterols and fatty acids of both the intestine and liver were also found to be labeled, confirming that extensive degradation of the molecule takes place within the intestine

When [U C^{14}] retinene was administered to rats, the pattern of release of $C^{14}O_2$ was, surprisingly, much the same, indicating that it too is readily oxidized in transit across the intestine Three quarters of the total radioactivity expired in the 24 hour period was collected in the first few hours

The interesting feature of these experiments is that the pattern of release of $C^{14}O_2$ into the expired air is completely different from that observed for [14 C^{14}] vitamin A when injected into rats (Wolf *et al*, 1957) In this case the $C^{14}O$ was liberated at a steady rate, 5 2 % of the dose being released within 24 hours The results of these tracer experiments suggest that these polyene compounds are subjected to a more intensive oxidative attack in crossing the intestine than when administered parenterally This is in keeping with the greater effectiveness of this organ in converting β carotene to vitamin A The above labeled compounds were administered in arachis oil, which contains about the right amount of vitamin E to protect small amounts of the provitamin

Some 10–15 % of the absorbed [U C^{14}] retinene was excreted in water soluble form in the 24 hour urine, which compares favorably with the value of 12 % obtained by Wolf *et al* (1957) for their specifically labeled vitamin A administered parenterally Bearing in mind the extent of the body's metabolic pool of small organic compounds, excretion of the radiocarbon at the level above must really mean that appreciable fragments of vitamin A are probably passed into the urine intact, such as a derivative of the ring moiety or side chain Prelog and Osgau (1952) have found β ionone derivatives in pregnant mares' urine

3 General Conclusions

The experiments with C^{14} labeled compounds show that when β carotene is administered orally in arachis oil, which contains about the right amount

of vitamin E for producing vitamin A it is metabolized to small fragments that can enter the metabolic pool This implies oxidative attack at more than one position in the chain The true nature of the conversion process is still far from clear but the work with synthetic β apocarotenoids tends to eliminate the normal β oxidation system as originally being responsible Although it has recently been demonstrated that β carotene can form a lipoprotein complex (Ganguly *et al*, 1959) during absorption across the intestine or *in vitro* (Shah, 1958) with everted intestinal sacs, the complete molecule would have to penetrate the mitochondrial membrane to reach the β oxidation enzyme system It would seem that oxidase enzymes are probably responsible for carotene degradation

The suggestions that have been made, that the β carotene can be attacked in the lumen of the intestine, point to a water soluble enzyme system being involved in the initial stages at least

If terminal oxidation does occur then the fragment larger than vitamin A can be degraded further to the vitamin The higher biological activity of the C_{25} β apocarotenoids compared to the others is striking and indicates that they provide better than the others the type of substrate which the A forming enzyme system requires The relatively low yield of vitamin A obtained from single doses of β apo 12′ carotenals compared to the excellent biological activity at low dose levels is difficult to understand Some vitamin A acid may be produced directly as well as vitamin A

The acid is difficult to trace in the animal body but is active in promoting growth although it cannot be reduced to vitamin A Alternatively, some of the bound forms of retinene such as those discovered by Kon and colleagues (Fisher and Kon 1959) may be formed

IV ALTERNATIVE SCHEME

The evidence from the study of the above synthetic intermediates suggests that the initial penetration of the β carotene molecule probably takes place nearer the center of the conjugated system than the end This immediately poses the question as to how it might be effected, and the following line of reasoning is tentatively suggested It is perhaps not unreasonable to assume that the long conjugated chain may be bent to render the molecule more susceptible to attack The various isomers of all *trans* β carotene that have a central double bond in the *cis* arrangement show selective absorption in the 320–360 mμ region the well known *cis* peak which is absent or very small in the all *trans* compound In 15 15′ *cis* β carotene, this peak is at its highest value with $E^{1\%}_{1cm}$ at 338 mμ = 1040, compared to values of 1765 and 1430 for the $\lambda\lambda_{max}$ at 450 mμ and 477 mμ, respectively

The latter maxima arise from the electron resonance involving 9 and 10 conjugated double bonds whereas absorption in the 300–360 mμ region

derives from the resonance mainly of 4 and 5 ethylenic bonds The *cis* configuration has increased the probability for resonance to occur along 4 or 5 ethylenic bonds on either side of the central bond in the molecule, where the overlap in the π electron fields has been weakened

The strength of the "oscillator" ($\int \frac{\nu_2}{\nu_1} \epsilon d\nu$) in conjugated polyene systems increases with the number of double bonds involved So when allowance is made for the fact that the strength of the "oscillators" in the *cis* peak region are only approximately two thirds those in the 450 mμ region, it seems that resonance involving only 4 or 5 double bonds of 15,15′ *cis* β carotene occurs just as frequently as that involving 9 or 10 double bonds

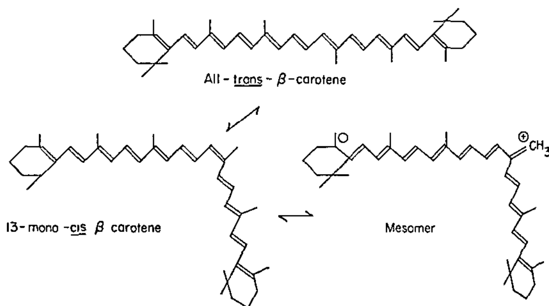


Fig 2 A suggested resonance state of 13 *cis* β carotene which might allow oxidative attack near the center

Now 15,15′ *cis* β carotene has only a biological activity of some 30% of the all *trans* isomer, so it is unlikely to be a key intermediate Neo β carotene B has greater biological activity with values approximately 50% that of the all *trans* compound It has been suggested that it is the 7′,13′ di *cis* compound on account of the height of the *cis* peak in its absorption spectrum The latter (log = 3 4) which is about one third the value (log = 9 5) at 450 mμ is relatively smaller than that for the 15,15′ mono *cis* compound and could perhaps really be the 13′ mono *cis* compound

One of the resonance states may be written as shown in Fig 2 The potential methylene group may then be oxidized or permit oxidative attack at a neighboring double bond close to the center of the conjugated chain The over all yield would still be one molecule of vitamin A from one molecule of β carotene, but fewer steps are involved in the process

It would therefore be interesting to observe for comparison the metabolites formed after the administration of single large doses of decapreno β carotene and dodecapreno β carotene (Karrer and Eugster, 1951) to determine whether higher vinylogs of vitamin A are formed directly *in vivo* The above hydrocarbons are biologically active and may form recognizable intermediates such as those mentioned above before being metabolized to vitamin A A study of the metabolism of these compounds might help to elucidate the nature of this conversion process, which may involve more than one route

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Absorption, Transport, and Storage of Vitamin A

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I INTRODUCTION

The transport in the animal body of lipid materials unlike that of water soluble metabolites offers a complex problem due to their inherent hydro phobic character Lipids should of necessity, be in such a state of disper sion in biological fluids that they can gain easy access to the cells In effecting such a dispersion, lipoproteins have been assigned a vital role Lipoproteins not only serve as carriers for lipids by making them soluble in biological fluids, but they are also, in fact, integral parts of the structures of individual cells and of their various cytoplasmic inclusions

Vitamin A is insoluble in water, but is readily soluble in fats In order to be biologically active it should normally be present in biological fluids dispersed at the molecular level and it is in this connection that the lipo proteins become indispensable in vitamin A metabolism

Discussions in this review will center around the role of lipoproteins in the absorption, transport and storage of vitamin A For other aspects the reader is referred to the earlier exhaustive treatments (Clausen, 1943, Sobel, 1952, Deuel, 1955, 1957 Moore 1957)

II Absorption from the Intestine

1 *Intraluminar Phase*

a Hydrolysis and Esterification of Vitamin A in the Gastrointestinal Tract Vitamin A alcohol or its esters do not appear to undergo esterification or hydrolysis in the animal stomach (Clausen, 1943, Thompson *et al*, 1949, 1950) Both forms appear in the lumen of the small intestine after oral administration This was first demonstrated by Gray *et al* (1940a), and since then it has been confirmed in several laboratories with different species of animals such as the rat (Clausen, 1943, Thompson *et al*, 1949, 1950), sheep, and calf (Eden and Sellers, 1950), using fish liver oil as the source of vitamin A More recent observations (Ganguly *et al*, 1959, Mahadevan *et al*, 1959a) have demonstrated that both the hydrolysis of vitamin A esters and the esterification of the free alcohol take place in the same locale when the synthetic, instead of the natural, vitamin is fed to rats

b Vitamin A Esterase Although Ganguly (1949) was able to obtain hydrolysis of vitamin A esters of fish liver oils by an extract of pig pancreas in an *in vitro* system consisting of rather crude dispersion media, the first clear demonstration of *in vitro* hydrolysis of vitamin A acetate by rat liver homogenate was probably by McGugan and Laughland (1952) This was immediately confirmed and extended by Ganguly and Deuel (1953) and by Ganguly (1954), who further demonstrated quantitative localization of the activity in the microsomal fraction of rat liver homogenate In chickens, however, the activity was found to be distributed between the nuclear and microsomal fractions of the liver homogenate (Krishnamurthy *et al*, 1958b)

The vitamin A esterase activity is widely distributed in the tissues of the rat But whereas blood and liver can act only on the lower fatty acid esters, other tissues, e g the pancreas and small intestine, are effective against both lower and higher fatty acid esters with almost equal efficiency (Krause and Powell, 1953, High *et al*, 1956, 1957, Seshadri Sastry *et al*, 1957, Laughland, 1958)

It was generally assumed that hydrolysis of vitamin A esters is brought about by a common lipase that acts on natural triglycerides Seshadri Sastry *et al* (1957), however, have come to different conclusions These investigators observed that, whereas an enzyme preparation from goat pancreas could rapidly hydrolyze triglycerides, simple aliphatic esters, and vitamin A esters, the acetone powder prepared from the same tissue retained its activity almost unchanged against the first two substrates, although it completely lost its activity against the vitamin A esters Further, the vitamin A esterase activity of rat liver was more easily reduced by various inhibitors and it was more labile toward heat and aging Moreover, the most recent studies of Seshadri Sastry (1960) have shown that the esterase, vitamin A esterase, and cholesterol esterase of chicken liver nuclei

can be separated by electrophoresis on agar blocks and by ammonium sulfate fractionation

Both hydrolytic and esterifying activities have been demonstrated *in vitro* with an extract of the pancreas (Mahadevan *et al*, 1960, Pollard and Bieri, 1960) and with the whole wall of the small intestine of the rat (Krause and Powell, 1953, High *et al*, 1956, Seshadri Sastry *et al*, 1957) It has also been shown to be present in the contents, as well as in the mucosae and muscles, of the small intestine of the same species (Mahadevan *et al*, 1960) It would thus appear that, although some of the intestinal enzymes may originate from the secretions of the pancreas, the intestine also elaborates its own enzyme activities

2 *Intracellular Phase*

Pioneering work in this direction was carried out by Gray *et al* (1940b) by analytical distillation of extracts of the intestinal walls of rats fed distilled fish liver oil Of the total vitamin A recovered 59% was in the alcohol form 220 minutes after dosing and this proportion rose to 82% after 400 minutes Clausen's observations (Clausen, 1943), on the other hand, showed that 48 5% of the total vitamin A recovered from the wall of the small intestine of rats receiving the vitamin orally was present as the free alcohol Later on, however, with the aid of adsorption chromatography for the separation of the ester and the alcohol, it has been consistently demonstrated that the ester form predominates in the wall of the small intestine of rats calves, chickens, and sheep, irrespective of whether fish liver oil, vitamin A alcohol its acetate, palmitate or aldehyde, or β carotene is used for feeding (Glover *et al* 1948 Eden and Sellers, 1950 Thompson *et al*, 1950, Plack 1959 Ganguly *et al*, 1959)

It has been shown that on further subdivision of the intestinal wall into mucosae and muscles about 75% of the total vitamin A is almost invariably present in the esterified form in both the tissue fractions (Ganguly *et al*, 1959) (Table I) It is interesting that several years ago Thompson *et al* (1950) had called attention to a similar percentage composition of vitamin A ester and alcohol in the whole wall of the small intestine of rats This is of particular significance, because as will be seen shortly vitamin A is transported from the intestine exclusively as its ester

It has been shown by resolution of only the ester fraction of the contents, mucosae and muscles of the small intestine the mesenteric lymph, and the postabsorptive blood and liver of rats fed either vitamin A alcohol acetate or palmitate that short chain fatty acid esters of vitamin A cannot be traced in the intestinal muscles, mesenteric lymph blood or liver regardless of the form fed After administration the acetate could not be detected in the muscles of the small intestine, though small amounts of it were present in the mucosae (Mahadevan *et al*, 1959a) This might be due to the acetate

being held mechanically on the mucosal cell surface Alternatively it might actually have entered the cell, to be hydrolyzed and re esterified there since both hydrolytic and esterifying systems for vitamin A have been found to be present in the mucosal cells Esterification in the intestine seems to take place specifically with long chain fatty acids (Mahadevan *et al*, 1960) Work of Krinsky (1958) has shown a similar fatty acid specificity in esterification of vitamin A in cattle retina also

Be that as it may, it seems quite clear that only the higher fatty acid esters, and not the lower ones, are transported beyond the mucosal cells This seems to be in full agreement with the earlier work of Gray *et al* (1940b) and Gray and Cawley (1942), who concluded from their results obtained with analytical distillation, that vitamin A in the liver of the rat

TABLE I

DISTRIBUTION OF VITAMIN A ESTER AND ALCOHOL AMONG THE CONTENTS MUCOSAL CELLS AND MUSCLES OF SMALL INTESTINE OF RATS AFTER FEEDING VITAMIN A ALCOHOL ITS ACETATE OR PALMITATE

Intestine	Alcohol (μg /gm)		Acetate (μg /gm)		Palmitate (μg /gm)	
	Ester	Alcohol	Ester	Alcohol	Ester	Alcohol
Contents	630 0	1920 0	1580 0	380 0	1020 0	447 0
Mucosal cells	17 0	6 2	28 5	11 0	20 0	9 2
Muscles	12 0	3 7	15 5	5 9	10 0	3 5

[a] Adapted from Ganguly *et al* (1959)

is esterified with one fatty acid, probably palmitic acid More recent work of Kaiser and Kagan (1956) also has shown that only long chain fatty acid esters of vitamin A are to be found in blood and liver of rats

The transport of vitamin A across the intestinal wall is energy dependent With the everted intestinal sac technique of Wilson and Wiseman (1954), its transport was shown to be greatly accelerated by extra glucose and/or adenosine triphosphate (ATP), whereas glycolytic inhibitors like sodium fluoride markedly retarded the process (Mahadevan *et al*, 1959c) Independently and simultaneously, Loran and Althausen (1959) have also shown that extra glucose activates the process of transport of the vitamin, whereas uncoupling agents like DNP inhibit it

III TRANSPORT

1 *From the Intestine through the Lymphatic System*

Drummond and his associates (1935) were probably the first to make the interesting observation that vitamin A is absorbed as its ester through the

lymphatic system They gave vitamin A orally to a patient with chylothorax and were able to recover the administered vitamin almost quantitatively from the collected lymph fluid About a decade later Popper and Volk (1944) noticed characteristic vitamin A fluorescence in the lacteals of rats within 25 minutes after feeding vitamin A Since these observations, reports appeared in quick succession demonstrating the lymphatic absorption of the vitamin Eden and Sellers (1948) and Goodwin and Gregory (1948) arrived at a similar conclusion with ruminants like bullocks and goats respectively In the following year Eden and Sellers (1949) and Thompson *et al* (1949) reported a marked increase in the concentration of the ester form in the mesenteric lymph glands of ruminants, such as bul-

TABLE II

RELATIVE APPEARANCE OF VITAMIN A ESTER AND ALCOHOL IN THE LYMPH FLUID OF PIGS AFTER VITAMIN A FEEDING

Time after dose (hr)	Alcohol (I U /100 ml)	Ester (I U /100 ml)
− 0.5–0	31	33
0–0.5	19	43
1.25–1.5	22	62
1.75–2.0	21	200
2.5–3.0	26	650
3.0–3.5	39	1370
3.5–4.0	31	1450
4.0–4.5	44	1540
4.5–5.0	55	1350
5.0–5.5	69	1700
5.5–6.0	55	1200

Adapted from Thompson *et al* (1950)

locks and sheep, and of nonruminants, such as pigs, as a result of feeding fish liver oil But the most convincing evidence for the absorption of vitamin A through the lymphatic route only and exclusively in the ester form came one year later (Thompson *et al* , 1950) Lymph cannulas were established in vitamin A deficient rats thereby preventing the emptying of lymph fluid into the systemic blood When such rats were given meals containing vitamin A or β carotene all the vitamin A absorbed could be collected with the lymph fluid that drained into a separate receiver while the liver still remained free of the vitamin The same workers drew pointed attention to a sharp and exclusive increase in the ester fraction alone in lymph fluids collected from pigs similarly cannulated and dosed (Table II) Furthermore, the transport of the vitamin through the portal route may be ruled out because no significant differences between the vitamin A concentrations of the portal and systemic blood could be detected after its intake by bullocks,

sheep, and rats (Eden and Sellers, 1948, 1949), by goats (Goodwin and Gregory, 1948), by pigs (Thompson *et al*, 1950), and by guinea pigs (Woyt kiw and Esselbaugh, 1951) It is thus clear that, although at the time of active absorption a certain proportion of the free vitamin is always present in the mucosae and muscles of the small intestine and in the mesenteric lymph fluid, it is transported from the intestine only as long chain fatty acid ester, and through *the lymphatic route alone*

2 In Blood

Clausen *et al* (1940) came to the conclusion that under fasting conditions nearly all the vitamin A in human plasma is present as the free alcohol Three years later Clausen (1943) showed that *ingestion or injection* of large amounts of the vitamin, or administration of alcohol (methanol or ethanol) brings about a marked increase in the ester concentration of the blood without changing its free vitamin A concentration Subsequent studies (Hoch, 1946, Hoch and Hoch, 1946) have shown that 10–17% of the blood vitamin A of normal human subjects is present as the ester with the rest in the free form, and that the oral intake of the ester or alcohol forms of vitamin A leads to an increase in blood in the ester fraction only These observations have since been repeatedly confirmed in humans (Popper *et al*, 1948, Week and Sevigne, 1950, Dost and Rind, 1956) and in rats and pigs (Thompson *et al*, 1949, 1950, Ganguly and Krinsky, 1953)

The vitamin A *alcohol level in the normal or fasting state is independent* of the size of the liver reserve of the ester or the free alcohol (Ganguly and Krinsky, 1953, High and Wilson, 1956) The vitamin A content of the blood is tenaciously preserved even at the expense of the last traces of the liver vitamin A, so that on prolonged depletion, although the vitamin may not be present in the liver, it can still be detected in the blood This latter tissue, however, ultimately loses its vitamin if the depletion is further continued (Glover *et al*, 1947, Ganguly and Krinsky, 1953, High and Wilson, 1956)

Vitamin A ester and alcohol are transported in normal and postabsorptive blood by separate lipoproteins and not by chylomicra The ester is probably carried by a low density lipoprotein *and the alcohol by a centrifugally* sedimentable protein (Dzialoszynski *et al*, 1945, Ganguly *et al*, 1952, Garbers, 1958, Garbers *et al*, 1958, Krinsky *et al*, 1958)

IV Storage in the Liver

1 Form of Vitamin A

Bacharach and Smith (1928) concluded that vitamin A is present in the esterified state in cod liver Several years later Reti (1935) also

arrived at the same conclusion regarding the vitamin A in the livers of fish, chickens, and various mammals It was left to Hickman (1936) to discover that small amounts of the free alcohol are present in fish liver oils along with considerably larger proportions of the ester Hickman and his colleagues (Gray *et al*, 1940b) subsequently confirmed that a small but definite amount of the free vitamin is always present in rat liver and arrived at the proportion of 95% ester and 5% alcohol

Since then it has been well established that the ester predominates in the liver, with small amounts of the alcohol always present In this connection the findings of Ganguly and Krinsky (1953) regarding the relative rates of deposition of the two forms of the vitamin in rat liver are of considerable interest In A deficient rats that had received a single oral dose of large amounts of vitamin A as fish liver oil, blood showed a typical sharp increase in its ester concentration at 5–7 hours after the dose, the alcohol showed a relatively smaller rise In the liver the free alcohol quickly reached a maximum value at 3–5 hours, and showed no further increase, whereas the ester continued to accumulate with time (Fig 1)

2 *Intracellular Distribution*

Reports on the localization of the vitamin within the liver cells have not been too uniform Earlier histochemical studies of Joyet Lavergne (1935) and of Bourne (1935) had indicated mitochondrial association, but the use of antimony trichloride for locating it inside a whole cell has been questioned by Popper (1944) Fluorescent microscopic studies, on the other hand, led to the conclusion that the vitamin is rather diffusely distributed within the cell (Popper, 1944) However, with the introduction of the technique of the differential centrifugation for the separation of cell constituents, it has been possible to obtain more definite information

In one of the earliest attempts with this technique, Goerner (Goerner, 1937–1938 Goerner and Goerner, 1938 1939) claimed that vitamin A is present in the mitochondria of rabbit liver cells isolated from suspensions of finely dispersed tissues according to the procedure of Bensley and Hoerr (1934) This method is open to criticism and has been superseded by a greatly improved procedure of Schneider and Hogeboom (1950) The method used for the estimation of the vitamin also is questionable insofar as it gives reactions with sterols as well (Moore, 1957) In preliminary experiments with one single rat, Collins (1952) obtained 3 4% of the vitamin in the nuclei, 20% in the mitochondria and 76 2% in the supernatant fluid (this fraction contained the microsomes also) The corresponding values reported by Powell and Krause (1953) were 15% 19 4% and 64 6% for rat liver Almost similar patterns of distribution were found in chicken liver also (Cowlishaw *et al*, 1957, Krishnamurthy *et al*, 1958b)

3 *Role of Kupffer Cells*

Based on histological studies, earlier workers attributed to the Kupffer cells an important role in vitamin A metabolism In vitamin A deficiency these cells degenerate (Frank, 1935, Uotila and Simola, 1938), whereas with an excessive intake of the vitamin, fatty infiltrations take place in the same cells (Nieman and Klein Obbink 1954) The technique of fluorescent microscopy has located most of the characteristic fluorescence given by vitamin A in the Kupffer cells of the liver (Popper and Greenberg, 1941) Blocking of the reticuloendothelial system resulted in a lowered deposition of the vitamin in the liver tissue (Lasch 1935, Lasch and Roller, 1936) Popper (1944) has ably reviewed the earlier work

TABLE III
INTRACELLULAR DISTRIBUTION OF VITAMIN A ESTER AND VITAMIN A ALCOHOL IN RAT LIVER

Fraction	Vitamin A ester		Vitamin A alcohol		Ratio of ester alco hol
	μg /gm liver	%[b]	μg /gm liver	%[b]	
Homogenate	376	—	5 3	—	70 9
Cream	262 4	86 2	2 3	52 3	114 1
Supernatant	13 8	4 5	0 9	20 4	15 3
Microsomes	12 9	4 2	0 7	15 9	18 4
Mitochondria	4 6	1 5	0 3	7 0	15 3
Nuclei	10 4	3 4	0 2	4 6	52 0

[a] Adapted from Krinsky and Ganguly (1953) Values averaged and recalculated
[b] Based on total recovered

However, the question of storage of vitamin A ester and alcohol individually does not appear to have received much attention until Glover and Morton (1948) made the interesting suggestion that probably the ester, dissolved in fats is stored in the Kupffer cells and the alcohol in the form of a protein complex, in the parenchymal cells The findings of Krishnamurthy and Ganguly (1956) essentially agree with this concept These workers showed that blocking of the reticuloendothelial system prior to oral administration of vitamin A caused nearly 50% reduction in the concentration of the ester, with practically no change in the alcohol level in the liver of A deficient rats (Table IV) It thus seems quite possible that the ester is stored in the Kupffer cells and the alcohol in the parenchymal cells of the liver

4 *Hydrolysis of the Liver Ester*

As early as 1940 Gray *et al* (1940b) had suggested that the free alcohol is probably the normal physiological form of vitamin A This was reaffirmed

by Morton (Glover *et al.*, 1947). The organism maintains the vitamin A alcohol content of its tissues at the expense of the liver ester (High and Wilson, 1956). This is only natural, since the animal has to meet its physiological requirements. But the possible mechanism of hydrolysis of the liver ester has not been fully understood. It has, however, led to some rather interesting speculation. The stored ester could not be hydrolyzed on prolonged autolysis of rat liver at room temperature (Glover *et al.*, 1947) or on incubation of the whole homogenate of rat liver (High *et al.*, 1957; Krishnamurthy *et al.*, 1957). This is to be expected, because, as already seen, the liver stores the higher fatty acid esters of vitamin A, and its vitamin A esterase is inactive against such esters.

TABLE IV

BLOCKING OF THE RETICULOENDOTHELIAL SYSTEM AND STORAGE OF VITAMIN A ESTER AND ALCOHOL IN RAT LIVER

Number of rats	Treatment	Blood (μg./100 ml. plasma)		Liver (μg./organ)	
		Vitamin A ester	Vitamin A alcohol	Vitamin A ester	Vitamin A alcohol
6	Negative control	—	—	10.2	1.4
6	Saline + 3000 μg. vitamin A	1.9	1.2	2238.3	56.0
6	India ink + 3000 μg. vitamin A	5.1	1.9	1168.8	54.3

[a] Adapted from Krishnamurthy and Ganguly (1956).

Several suggestions have been made to explain the mechanism of hydrolysis of the liver ester. Glover *et al.* (1947) considered that this hydrolysis might be due to a lipase present in the Kupffer cells. But the liver does not seem to have any significant lipolytic activity. Another suggestion was that the hydrolysis might be brought about by the serum esterase (Krause and Powell, 1953), but Krishnamurthy *et al.* (1957) have found the serum esterase inactive against the stored ester. According to High *et al.* (1957), the higher fatty acid esters of vitamin A might be broken down to the shorter state in the liver before they are hydrolyzed in the same tissue. However, the mechanism of the degradation of the long chain fatty acid esters of the vitamin is not quite clear. Yet another possibility was suggested by Krishnamurthy *et al.* (1957), who were able to demonstrate that a small proportion of the ester is always present not only in the normal circulating blood, but even in the blood of fasting rats. The blood ester of rats could not be hydrolyzed by blood or liver, but was readily hydrolyzed by other tis

sues, e g pancreas and small intestine It was, therefore, proposed by them as a working hypothesis that during the course of its circulation the ester present in normal circulating blood undergoes hydrolysis in the extrahepatic tissues to return to the liver as the free alcohol It is not unlikely that this small amount of the ester of the fasting blood is carried from the Kupffer cells of the liver by low density lipoproteins (Krinsky *et al*, 1958)

V Lipoproteins in Transport and Storage of Vitamin A

It is well known that whereas the liver can accumulate large amounts of esterified vitamin A, the amount of the free alcohol that can be stored there is relatively limited It is evident from Fig 1 that within 3–5 hours after vitamin A administration, the free alcohol level in the liver of the A-deficient rats had reached a state of saturation whereas the ester was still being deposited even after 18 hours This would baffle our existing knowledge of enzyme action, because (assuming that some mechanism exists in the liver to hydrolyze the incoming ester) one would normally expect at least some proportional increase of both forms with time

Similarly it would appear from Table III that the two forms were not distributed in any uniform manner among the various cell fractions of the liver homogenate The ester was mostly (86 2 %) concentrated in the "cream" fraction, whereas the free alcohol, in addition to being present largely (52 3 %) in the same fraction, was found in considerable amounts in the microsomal and supernatant fractions also These two latter fractions together contained 36 3 % of the alcohol but only 8 7 % of the ester One would normally expect proportional distribution of both the forms, if one were to assume their solubility in lipid materials as the criterion of the distribution

These two observations were explained by assuming that the free and esterified vitamin A are associated with different proteins It was suggested that the protein that binds with the alcohol gets saturated easily and tends to remain saturated (Ganguly and Krinsky, 1953, Krinsky and Ganguly, 1953) It has already been noted that Krishnamurthy and Ganguly (1956) were able to show that the free and esterified vitamin A are actually stored in two different types of cells of the liver—the parenchymal and Kupffer cells, respectively

Speculations regarding the possibility of binding of carotenoids and vitamin A with proteins in animal tissues are not new Palmer and Eckles (1914) had even claimed to have isolated from bovine serum a carotene protein complex which they had named "caroto albumin" Observations of Zechmeister (1937) Lovern *et al* (1937), Pett and Le Page (1940), Lawrie *et al* (1941), and Dzialoszynski *et al* (1945) have all led to the conclusion that carotenoids and vitamin A are associated with proteins in

animal tissues As a matter of fact, the method of Kimble (1939), which is most commonly used for extraction of blood lipids, consists of prior denaturation of proteins by ethanol followed by extraction with solvents like petroleum ether

However, with improved knowledge of lipoproteins, an increasingly important role is being attributed to these macromolecules in the transport and storage of lipid materials in the animal body (Frazer, 1949, Oncley *et al*, 1950) Though Machebouf (Machebouf and Rebeyrotte, 1949) probably ranks as the pioneer in the isolation of the first lipoprotein "Acid Cenapse," most of our present knowledge concerning the physicochemical properties and the biological importance of lipoproteins has come from the Harvard School

Using methods commonly employed for the isolation and identification of lipoproteins, Krishnamurthy *et al* (1958a) were able to demonstrate that the esterified and free vitamin A are associated with different lipoproteins in rat liver The same workers were not able to give direct evidence for the specific nature of the binding of the ester and the alcohol with separate proteins, as no such binding could be effected *in vitro*, nevertheless, several observations would give indirect support to a concept of the specific nature of the binding of lipoproteins with carotenoids, vitamin A ester, and vitamin A alcohol

Mehl (1944) had first noticed that the carotenoids of human plasma are concentrated in one protein fraction, and subsequent work of Oncley *et al* (1950) has shown that actually β lipoprotein contains most of the carotenoids of human serum In chicken plasma, vitamin A ester and alcohol were shown to be associated with different lipoproteins (Ganguly *et al*, 1952) Similarly vitamin A and carotenoids are associated with different protein fractions in human serum (Hack, 1956) and in bovine serum (Erwin *et al*, 1959) In rat blood vitamin A ester is transported by a low density lipoprotein and the alcohol by a centrifugally sedimentable protein (Garbers, 1958, Garbers *et al*, 1958) Furthermore, a carotenoid protein complex has been isolated from chicken liver by Mahadevan *et al* (1959b) But, probably the most compelling evidence was produced by Krinsky *et al* (1958) Table V clearly shows that in postabsorptive human blood the chylomicra are practically free from fat soluble compounds like the carotenoids, vitamin A ester, and vitamin A alcohol This observation is of particular significance in relation to the mechanism of absorption of lipid materials from the intestine Not only are these fat soluble compounds absent from the chylomicra, where they would normally be expected, but they even fractionate with different lipoproteins

Let us take the case of vitamin A As already discussed, it is absorbed from the intestine as higher fatty acid ester Krinsky *et al* (1958) have now

shown that in postabsorptive blood this ester is actually present in the low density S_f 10–100 lipoproteins, and not in the chylomicra This can only mean that in the intestine some unknown mechanism takes it out of fat solution and esterifies it with higher fatty acids It is then transported by the low density lipoproteins, which probably act as carriers, and is ultimately deposited in the Kupffer cells of the liver The free alcohol, which is the normal form in blood, on the other hand, although it fractionates with the albumin is not actually bound to the blood albumin but to some other protein that fractionates with the albumin (Krinsky *et al*, 1958) There is thus a good case here for the concept of a carrier mechanism for lipids in transcellular transport as well as in their transport from one tissue to another during normal circulation

TABLE V

DISTRIBUTION OF VITAMIN A ALCOHOL VITAMIN A ESTER AND CAROTENOIDS IN HUMAN SERUM DURING ACTIVE ABSORPTION [b]

Serum fraction	Vitamin A alcohol (%)	Vitamin A ester (%)	β Carotene and lycopene (%)	Lutein (%)
Chylomicra	5 3	7 5	0	6 4
S_f 10–100	3 9	79 4	0	9 1
S_f 3–9	20 2	8 6	78 3	50 3
Other serum proteins	70 6	4 4	21 7	34 3

Adapted from Krinsky *et al* (1958)
[b] Three hour sampl

Mention should also be made of the possibility that there is a certain amount of stereospecificity in this mechanism of protein (or lipoprotein) binding The classic demonstration by Wald (1953) of such a stereospecificity in the visual system would probably be a most convincing example The very recent results of Plack (1959) would also appear to indicate the possibility of a similar stereospecificity in the absorption mechanism

VI CONCLUDING REMARKS

Lipoproteins have assumed considerable importance in the metabolism of vitamin A During active absorption a low density lipoprotein probably transports it from the intestine as its ester and deposits it as such in the Kupffer cells of the liver, while another lipoprotein carries it from the parenchymal cells to different tissues as the free alcohol for metabolic needs

In spite of intensive work for nearly half a century, our knowledge regarding the exact mechanism of absorption of lipid materials from the

intestine is far from complete An approach based on lipoproteins may lead to a better understanding of this complex problem

The brilliant work of Wald has led to a clearer understanding of the function of vitamin A in the visual system, in spite of this, the general mode of action of the vitamin still remains a mystery The function of this vitamin in the maintenance of the structural integrity of lipoproteins of epithelial cells and of other tissues may be a fruitful line of work

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Metabolic Tranformations of Vitamin A

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I Introduction

The approach to a study of the metabolic transformations of vitamin A has been from two different directions first, to find a functional form of vitamin A, since circumstantial evidence makes it seem likely that is is not vitamin A itself but a derivative thereof which is the active form So for instance, as discussed by Moore (1957a) the reserve stores of vitamin A disappear long before deficiency symptoms are seen, and much more vitamin A is required for storage than for growth At least 100 I U of vitamin A disappear when administered to a depleted rat, before storage begins (Davies and Moore, 1948) It seems as if vitamin A, when fed to deficient rats, is first transformed into an active derivative which is used for growth promotion and tissue maintenance When this derivative reaches its proper level, vitamin A itself appears in the blood and is ultimately stored in the liver An excellent discussion of the present state of knowledge of this unknown derivative and the "alleged hidden forms" of vitamin A is given by Moore (1957a)

The second approach is to determine the fate of administered vitamin A with regard to disposal by the organism of excess vitamin and disappearance after it has fulfilled its function (turnover)

II Active Derivatives of Vitamin A

A number of derivatives of vitamin A have been made by synthesis and others occur naturally, biological activity is shown in Table I, compiled by Moore (1957b) There is no evidence as yet that the following synthetic active derivatives vitamin A acid, vitamin A ethers, vitamin A phosphate,

and vitamin A hydrocarbon (axerophthene) are formed by metabolic reactions from vitamin A

On the other hand, the following derivatives of vitamin A have been found in nature The all *trans* isomer is the most active form systemically Enzymes exist in liver that can interconvert the all *trans* vitamin A or any *cis* isomers into an equilibrium mixture of *cis* and *trans* isomers, independent of the composition of the isomers administered (Ames *et al*, 1957) Other investigators (Murray *et al*, 1959) report storage primarily as the all *trans* form after feeding of the all *trans*, and of a *cis* form after feeding

TABLE I

EFFECT OF VARIOUS CHEMICAL CHANGES ON THE BIOLOGICAL ACTIVITY OF VITAMIN A[a]

Process	Product	Approximate activity (all *trans* vitamin A = 100)
Esterification	Natural or artificial esters	100
Oxidation	Aldehyde	100
Cis isomerism	*Cis* isomers	23-75
Ether formation	Phenyl or methyl ethers	10-100
Dehydrogenation	Vitamin A_2	30
Loss of oxygen	Axerophthene	10
Ketone formation	C_{21} ketone	10
Demethylation	Norvitamin A	10
Addition of CH_2	Homovitamin A	1 5
Dehydration	Anhydrovitamin A	0 4
Oxidation	Epoxide	0
Hydrogenation	Dihydrovitamin A	0

[a] From Moore (1957b)

of that *cis* isomer An enzyme was isolated from cattle retinas by Hubbard (1956) which isomerizes all *trans* vitamin A aldehyde to the 4 mono *cis* isomer (numbering according to Quaife (1954) and *Chemical Abstracts*, 1955 Index, starting with C_1 at the hydroxyl end of vitamin A) Plack (1959), on the other hand, showed that injected 4 mono *cis* vitamin A is predominantly converted to the all *trans* form, the conversion taking place in the small intestine The biopotencies of the geometrical isomers have been investigated and are shown in Table II, from the excellent review of the subject by Ames (1958)

As shown in Table II, all *trans* vitamin A aldehyde (retinene), as well as its *cis* isomers, has systemic vitamin A activity Bliss (1949), after the discovery by Wald and Hubbard (1949) of the requirement of diphospho

pyridine nucleotide for the oxidation of vitamin A to its aldehyde, found that liver alcohol dehydrogenase could interconvert the two compounds The ubiquity of this enzyme can account for the results of Glover *et al* (1948), who demonstrated the reverse reaction—the reduction of vitamin A aldehyde to vitamin A—to take place in intestine, liver, and other tissues Only one reported instance exists, however, of the existence of vitamin A aldehyde outside the retina Plack *et al* (1958) found it to be bound to protein in fish eggs

Vitamin A_2 (I) (Vitamin A with an added conjugated double bond in the β ionone ring) has about 30% of the biological activity of vitamin A It is formed from vitamin A in livers of at least some freshwater fishes

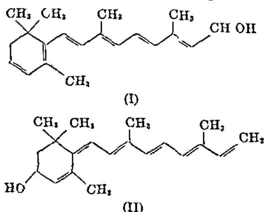
TABLE II

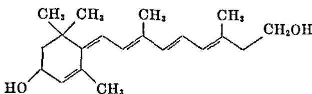
Biological Potency of the Geometric Isomers of Vitamin A[a]

Isomer	Isomeric acetates relative molar biopotency	Isomeric aldehydes relative molar biopotency
	(%)	(%)
All *trans*	100[b]	91
2 Mono *cis*	75	93
6 Mono *cis*	21	19
2 6 Di *cis*	24	17
4 Mono *cis*	24	47
2,4 Di *cis*	15	31

[a] From Ames (1958)
[b] By definition

(*Gambusia holbrooki*, Grangaud and Moatti, 1958), though the change back to vitamin A has not as yet been found to take place (Shantz *et al*, 1946) An anhydroform of vitamin A_2, with a postulated structure (II),



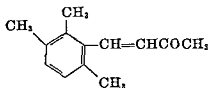


(III)

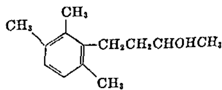
was found in freshwater fish liver (presumably derived from vitamin A_2) by Balasundaram *et al* (1958) This compound had biological vitamin A activity When fed to rats, it was converted to a rehydrovitamin A_2 (postulated structure III), which was stored as the free alcohol or ester in liver This is the first demonstration of the occurrence in liver, and of biological activity, of a β ionone ring substituted derivative of vitamin A This finding may have some importance in relation to the postulated active derivative of vitamin A Since vitamin A acid, vitamin A ethers, and vitamin A hydrocarbon (axerophthen) all show some activity, it could be concluded that, whereas the conjugated chain of four double bonds is essential for activity, the functional group at the end is not One could visualize a "fit" of the all *trans* chain to a specific protein molecule, without necessarily a participation of the end group Then the introduction of a new functional group in the β ionone ring may be required to form the reacting part of the molecule, after attachment of the chain to the enzyme protein

III Degradative Metabolism of Vitamin A

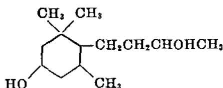
Prelog *et al* (1948, Prelog and Osgan 1952) discovered a series of β ionone derivatives in pregnant mares' urine which are undoubtedly derived from carotenoid compounds, probably vitamin A (see structures IV–VII)



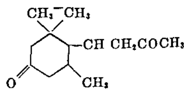
(IV)



(V)



(VI)



(VII)

Aromatization of the β ionone ring has taken place, with consequent migration of the methyl group in compounds (IV) and (V), whereas (VI) and (VII) show substitution of a hydroxyl and a keto group, respectively, in those positions found substituted in vitamin A_2 derivatives (II and III)

by Balasundaram *et al* (1958) It would be tempting to speculate along such lines as that vitamin A, with substitution of a hydroxyl group in the β ionone ring as in (III), may be an intermediate in an aromatization of its ring Such a phenolic compound would then be closely related to vitamins K E and ubiquinone In fact, Grangaud *et al* (1958) reported on the vitamin A like (antixerophthalmic) activity of an ester of just such a ring hydroxylated compound, 4′ hydroxy 3′ ketoretinene

With the advent of the use of tracers in biology, investigations of the degradative metabolism of vitamin A, as well as the search for an active form, became more direct and feasible

The first work was done with biosynthetic (uniformly labeled) β carotene-C^{14} by Willmer and Laughland (1957) The compound was adminis-

TABLE III

DISTRIBUTION OF C^{14} IN NONSAPONIFIABLE FRACTION AS A PERCENTAGE OF TOTAL ACTIVITY RECOVERED IN THE TISSUES

Tissue	Hours after dosing						
	2	4	6	10	16	22	28
Adrenal	20 80	17 49	24 47	39 00	28 08	17 36	20 27
Intestine	44 40	44 10	47 01	23 07	7 66	7 98	0 00
Kidney	2 60	2 00	3 13	1 90	4 17	4 32	6 96
Liver	15 47	31 27	23 41	34 16	52 02	67 51	67 29
Blood	16 73	5 16	1 98	1 84	3 07	2 83	5 48

[a] From Willmer and Laughland (1957)

tered to rats by stomach tube and radioactivity distribution was determined over a 28 hour period As might be expected, total radioactivity in nonsaponifiable fractions was highest in liver and intestines but adrenal glands followed closely More than 90% of the activity in liver or adrenal nonsaponifiable was accounted for as vitamin A During the early stages, activity was found to be higher in intestinal walls and blood, later to be redistributed into other organs (Table III) The adrenal gland, again was unique in rapidly taking up high activity and maintaining it throughout the experimental period Again in specific activity (radioactivity per weight of tissue), the adrenal gland led for the whole period, at 10 hours, for instance being 255 times that of liver The earlier demonstration by Popper and Greenberg (1941) of a concentration of vitamin A in the adrenal cortex is thus here confirmed, and support is given to the theory of an involvement of vitamin A in adrenal cortex function (Wolf *et al* 1958)

Degradative metabolism of the β carotene-C^{14} appeared to take place,

as 12% of the radioactivity was found in expired CO This was thought to be derived mainly from vitamin A rather than carotene, since it only appeared in the later hours, when most of the conversion of carotene to

TABLE IV

RADIOACTIVITY DISTRIBUTION

Material	Rat I		Rat II	
	Activity $\mu c \times 10^{-2}$	% of Dose	Activity $\mu c \times 10^{-2}$	% of Dose
Urine				
Ligroin extract	0 058	0 02	3 895	0 91
Water soluble residue	33 943	11 67	20 373	4 76
Feces				
Nonsaponifiable	0 667	0 23	0 000	0 00
Saponifiable and water soluble residue	64 235	22 15	0 000	0 00
Carcass				
Nonsaponifiable	28 710	9 90	28 820	6 74
Saponifiable and water soluble residue	19 314	6 66	92 971	21 73
Liver				
Nonsaponifiable	27 028	9 32	3 100	0 71
Saponifiable	9 628	4 64	—	—
Water soluble residue	3 335	1 61	—	—
Intestine				
Nonsaponifiable	6 206	2 14	—	—
Saponifiable	3 857	1 33	—	—
Water soluble residue	10 643	3 67	—	—
Blood				
Nonsaponifiable	0 145	0 05	—	—
Saponifiable and water soluble residue	0 435	0 15	—	—
Kidney				
Nonsaponifiable	1 102	0 38	0 813	0 19
Skin				
Nonsaponifiable	5 626	1 94	1 284	0 30
Eyes				
Nonsaponifiable	0 008	0 009	—	—

[a] Duration of experiment rat I 24 hours rat II 14 hours Weights rat I 205 gm rat II 315 gm Vitamin A C^{14} injected intraperitoneally rat I 2 80 mg (? 90 μc) rat II 4 14 mg (4 28 μc)

vitamin A had taken place A similar conclusion may be drawn from the finding of a rather high level of radioactivity in the saponifiable fraction, which would include fatty acids as well as water soluble compounds In this fraction again, the adrenals led in total and specific activity, the latter, at 6 hours, being 220 times that of liver, showing an active metabolic system in the adrenal, possibly connected with function

This very careful study of β carotene C^{14} metabolism was repeated, though less thoroughly, by Krause and Sanders (1957), who found less degradative metabolism to CO_2 or fatty acids Similar results were obtained by Fishwick and Glover (1957), who fed carotene C^{14} These authors found a higher specific activity in liver steroids than fatty acids, and conclude that, at least for carotene, breakdown may be into units larger than acetate which could enter the pathways of steroid preferentially to that of fatty acid biosynthesis

The degradative metabolism of vitamin A itself was first investigated by Wolf and co workers (1957) These investigators after synthesis of vitamin A 2 C^{14}, and intraperitoneal injection of it in colloidal suspension,

TABLE V
PAPER CHROMATOGRAPHY

Material	R_f in solvent systems			
	Butanol	Butanol saturated with water	Water saturated with butanol	93% Toluene 7% butanol
Urine water soluble (WS)	0 75–0 80	—	—	0 00
Urine water soluble (WS) acetylated derivative	0 68	—	—	0 00
Urine water soluble (WS) DNP derivative	—	—	—	0 85
Urine ether soluble (WES)	0 87–0 92	0 92	0 82	0 86–0 89
Urine ether soluble (WES) acetylated derivative	0 88	—	—	0 90
Urine ether soluble (WES) DNP derivative	0 87	0 92	0 00	0 86

accounted for about 5% of the radioactivity in 24 hours in expired CO_2 The distribution in other fractions is shown in Table IV The high percentage in carcass and liver fatty acids and water soluble fractions is noteworthy The radioactive water soluble fraction excreted in urine was further investigated It was found to consist of two distinct compounds, separable by paper chromatography (Table V), one being ether soluble as well as water soluble (WES, 33%), the other ether insoluble and water soluble (WS, 67%) Responses to spot tests for functional groups on paper chromatograms are shown in Table VI, radioactivity coincided with the colored spots obtained From these reactions it was concluded that WES contained unsaturation, hydroxyl group(s) and an aldehyde carbonyl group WS showed evidence of a keto group, upon acetylation it became ether soluble, this being evidence for the presence in it of one or more hydroxyl groups Dinitrophenylhydrazone derivatives could be made from

WS and WES, and paper chromatographed (Table V) A larger quantity of WS was obtained by injecting nonradioactive vitamin A into several animals and extracting the metabolites from urine by the method elaborated with the use of the isotopic compounds The dinitrophenylhydrazone of WS was recrystallized with the same derivative of radioactive WS as tracer Elementary analysis showed it to be a ketone of the approximate composition $C_{11}H_{14}O_4$ Spectrophotometric evidence indicated the carboxyl group of an ester and a nonconjugated keto group In similar tests, WES appeared to be a conjugated aldehyde Upon injection of labeled vitamin A into rats, Varandani (1959) found no trace of water soluble metabolites in liver, although they occurred in urine Their site of formation, therefore, is probably in another organ

From the above evidence it is difficult to draw any conclusions about

TABLE VI
FUNCTIONAL GROUP TESTS ON PAPER CHROMATOGRAMS

Reagents	Groups tested for	Urine, water soluble (WS)	Urine ether soluble (WES)
$KMnO_4$	OH, —C=C—	(−)	(+)
$KMnO_4/NaIO_4$	HO—C—C—OH	(−)	(+)
$C(NO_2)_4$	—C=C—	(−)	(+)
Dinitrophenylhydrazine	C=O	(+)	(+)
$AgNO_3/NH_3$	HC=O	(−)	(+)

the identity of these metabolites, and the metabolic pathway by which they have been derived from vitamin A Since vitamin A was labeled in position 2, one could surmise that they represent that part of the chain of the vitamin A molecule split off in the formation of the β ionone derivatives (IV–VII) identified as metabolites, as described above

In a study of the transport of vitamin A *2* C^{14} in serum of rats, Garbers *et al* (1960) showed that, whereas vitamin A was bound to α_1 globulin, a second radioactive component was detectable, associated with the α_2 globulin fraction and amounting to 23–29% of the total serum radioactivity This compound, as distinct from vitamin A, was not extractable with petroleum ether after ethanol denaturation of the protein, nor was it dialyzable The rats were all either vitamin A deficient or in a state marginal in vitamin A supplies, with a normal level of vitamin A in blood serum The compound could be either an active derivative or a degradation product of vitamin A, possibly related to the water soluble metabolites found by Wolf *et al* (1957) and Garbers *et al* (1960) to be excreted in urine to the extent of 7–12% of the administered dose

The vitamin A derivative found in serum may perhaps be identical with that formed when vitamin A is incubated with hemolyzed red blood cells (Pollard and Bieri, 1958) Vitamin A was found to disappear, but the products formed have not been identified The reaction involved seems to be an oxidation reaction and may perhaps be related to the action of lipoxidase like enzymes, known to attack carotenoids and mediated through peroxides Le Gallic (1947), also reported on a component of serum with vitamin A biopotency, but not responding to the Carr Price color reaction of vitamin A, which may be related to the metabolites mentioned above

TABLE VII

SUBCELLULAR DISTRIBUTION OF C^{14} LABELED VITAMIN A[a]

Fraction	% of total in cells
Nuclei	10 8
Mitochondria	40
Microsomes	23
Supernatant fraction	32

[a] Total activity injected 2 64 μc 3 times over 36 hours

TABLE VIII

RADIOACTIVITY OF SUBCELLULAR FRACTIONS

	Ether extractable (c p m)	Ether extractable after alcohol treatment (c p m)	Ether extractable after KOH treatment (c p m)
Supernatant	205 600 (61)	47 400 (16)	34 000 (23)
Mitochondria	354 000 (97)	1 700 (0 3)	9 000 (2 7)
Microsomes	190 000 (93)	8 000 (4)	6 200 (3)

The figures in parentheses indicate the percent of total radioactivity of each subcellular fraction

Varandani (1959) investigated the subcellular distribution of radioactivity after injection of labeled vitamin A into a normal rat Table VII shows that radioactivity appears in all cell fractions, whereas from Table VIII it can be seen that this activity is all ether extractable, and therefore probably unchanged, free vitamin A except for the supernatant fraction in which much of it is firmly, probably covalently bound One might suppose then that a functional metabolite of vitamin A occurs in the liver supernatant fraction, probably bound to protein

In order to reduce the number of uncontrollable variables, an *in vitro* system was sought that would metabolize vitamin A Wright (1960) incubated mitochondrial and microsome supernatant fractions of pig adrenal

gland homogenates with vitamin A-*1′,9 C^{14}* acetate (obtained through courtesy of Hoffmann-LaRoche Co) The adrenal was chosen in view of the functional involvement of vitamin A in adrenal steroid biosynthesis (Wolf *et al* , 1958) Under aerobic conditions, a large amount of radioactivity (in the mitochondrial preparations up to 60 %) was lost, presumably as CO_2 Esterase activity was present in both mitochondrial and microsome supernatant fractions, up to 50 % of the incubated vitamin A acetate being hydrolysed to free vitamin A in the microsome supernatant fraction Under anaerobic conditions, similar results were obtained, except that there was almost no loss to CO_2 Little esterase activity was detected in liver homogenate mitochondrial preparations, as distinct from adrenal (Table IX)

This work was badly hampered by the large amounts of polar and water

TABLE IX

VITAMIN A ESTERASE ACTIVITY IN CELL FRACTIONS

Fraction	Remaining ester[b]	Vitamin A alcohol[b]	More polar compounds[b]
Adrenal microsome and supernatant	11 1	50 0	34 8
Adrenal mitochondria	31 4	36 0	27 8
Liver mitochondria	49 3	8 7	18 0
Boiled mitochondria, control	69 0	0 0	19 3

[a] Each incubation contained 0 1 *M* phosphate buffer pH 7 4 0 25 *M* sucrose 0 03 *M* nicotinamide 0 004 *M* $MgCl_2$ 1 mg ATP 1 mg DPNH 0 1 μc vitamin A C^{14} acetate (15 μg) Separation of the reaction products was on deactivated alumina columns

[b] Values are percentages of recovered activity

Eluted from deactivated alumina with ethanol

soluble radioactive artifacts produced on incubation of the radioactive vitamin with boiled enzyme controls (Tables IX and X), even anaerobically, or simply during fractionation of the reaction products on deactivated alumina columns

Since Dowling and Wald (1960) demonstrated the metabolic activity of vitamin A acid, a search was made for this compound among the products of the incubations described above None was detected, nor could radioactivity be trapped in nonradioactive vitamin A acid when incubated with adrenal or liver homogenates and radioactive vitamin A

As indicated in the Introduction, after injection of a small amount of vitamin A into a deficient rat, no vitamin A is detectable in the tissues where it normally occurs, presumably owing to a transformation of it to an active form In order to obtain more information concerning the character and identity of this derivative, small amounts (75 μg , 218 I U , 225 μg , 690 I U) of vitamin A C^{14} acetate were injected into deficient rats

The results of this experiment are shown in Table XI It is evident that most of the radioactivity in the organs is not in the free state, but bound in such a way as to be released by protein denaturants One may then con-

TABLE X

FORMATION OF WATER SOLUBLE ARTIFACTS FROM VITAMIN A C^{14} ACETATE

Fraction	Aerobic			Anaerobic		
	Water soluble	Bicarbonate soluble	Alkali soluble	Water soluble	Bicarbonate soluble	Alkali soluble
Adrenal microsomes and supernatant	18 0	1 7	2 5	5 9	0 5	2 7
Adrenal mitochondria	17 0	2 7	4 3	6 1	1 0	3 9
Boiled mitochondria control	20 0	3 7	2 6	8 7	0 5	1 5
Buffer alone	Not investigated			6 9	—	2 5

Buffer and additives as for Table IX Values are percentages of recovered radioactivity

TABLE XI

DISTRIBUTION OF RADIOACTIVITY DERIVED FROM VITAMIN A 1 9 C^{14} ACETATE AFTER INJECTION INTO DEPLETED RATS

Organ	Experiment	Total activity (d p m)	Ether extractable (d p m)	Ether extractable after alcohol treatment (d p m)	Ether extractable, after alkali treatment (d p m)
Liver	1	27 150	2 640	10 700	13 800
	2	72 596	Not determined	Not determined	Not determined
Kidneys	1	6 800	1 070	3 160	2 570
Colon	1	1 720	None	595	1 125
	2	5 040	360	1 230	3 450
Heart	1	403	None	236	117
Adrenals	1	None	—	—	—
	2	2 480	None	2 600	None

In experiment 1 1 1 × 10 d p m (75 µg) were injected 4 hours prior to sacrifice in experiment 2 3 3 × 10^6 d p m (2 5 µg) were injected 20 hours prior to sacrifice

clude that the vitamin or metabolites thereof can be bound to lipoprotein (released by alcohol treatment) or to protein by covalent links (released by alkali treatment) It was especially interesting to note that in liver and kidneys about one half, in colon almost all of the recovered radioactivity

was protein bound In view of the known involvement of vitamin A in mucopolysaccharide biosynthesis in colon (Wolf and Varandani, 1960), one might speculate that colon in the deficient animal is among the first organs to bind some of the active metabolite of the vitamin, and thus restore to normal its depleted *biosynthetic activities* In the adrenals, on the other hand, no radioactivity was found 4 hours after injection of the labeled vitamin It obtained its share only much later, and here all of the activity was bound loosely (possibly to lipoprotein), thus showing a difference in the manner of utilization This may be a reflection of a different active form and mode of action in the two organs

Many of the above conclusions are as yet speculative, and the data are to be considered preliminary Work is under way to investigate further the character and identity of the metabolities of vitamin A and the manner in which they are bound

IV Conclusions

The search for a derivative of vitamin A which is the active form metabolically, has not yet proved fruitful The metabolic transformations of vitamin A so far detected—to the *cis* isomers, to the aldehyde, to vitamin A and its anhydro and rehydro forms—have not yielded compounds with greater biological activity than all *trans* vitamin A itself

Aromatization of, and introduction of oxygen functions into, the *β* ionone ring seem to occur, and a *β* ionone ring hydroxylated derivative of vitamin A possesses some vitamin A activity However, any conclusions from these results regarding an active derivative of the vitamin is purely speculative

Vitamin A is actively degraded to an appreciable extent into smaller fragments, some of which are converted into carbon dioxide and fatty acids, others excreted in urine as water soluble compounds A protein bound derivative is formed and detectable in serum, which may be an active derivative or a breakdown product of the vitamin

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The Visual Function of the Vitamins A

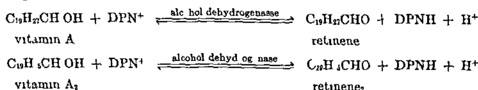
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The retinas of most vertebrate eyes possess two kinds of light receptor rods for vision in dim light and cones, for vision in bright light and for color vision Each of these organs contains a photosensitive pigment, which bleaches on exposure to light Some aspect of this process leads to a nervous excitation, which transmitted from one neuron to another along the optic pathways to the brain ends in exciting visual sensations

Four such pigments are widely distributed among vertebrate eyes rhodopsin and porphyropsin in rods and iodopsin and cyanopsin in cones Each is composed of a polyene chromophore united with a specific type of protein, called an opsin Two chromophores are known retinenes 1 and 2, the aldehydes of vitamins A_1 and A_2, and two main types of opsin, one found in rod outer segments the other in cones The two retinenes combine with the two kinds of opsin to yield the four main classes of visual pigment

Interconversions between the retinenes and the corresponding vitamins A are catalyzed by the enzyme, alcohol dehydrogenase, and the coenzyme diphosphopyridine nucleotide (DPN) an enzyme system widely distributed among animal tissues and present also in high concentration in the retina (Wald and Hubbard, 1948–1949, Bliss, 1949) The process involves only the transfer of hydrogen between the coenzyme and the terminal group of vitamin A or retinene



$$\underset{\text{vitamin A}}{C_{19}H_{27}CH_2OH} + DPN^+ \underset{}{\overset{\text{alcohol dehydrogenase}}{\rightleftharpoons}} \underset{\text{retinene}}{C_{19}H_{27}CHO} + DPNH + H^+$$

$$\underset{\text{vitamin } A_2}{C_{19}H_{25}CH_2OH} + DPN^+ \overset{\text{alcohol dehydrogenase}}{\rightleftharpoons} \underset{\text{retinene}_2}{C_{19}H_{25}CHO} + DPNH + H^+$$

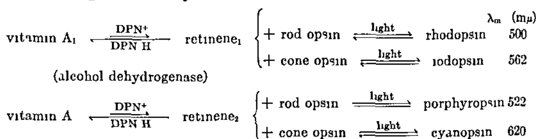
The bleaching of a visual pigment ends by splitting retinene from opsin The retinene is then reduced by the alcohol dehydrogenase system to vitamin A To resynthesize a visual pigment, vitamin A must be reoxidized to retinene The equilibrium of the alcohol dehydrogenase system, however,

* The original investigations from our laboratory were supported in part with grants from the Rockefeller Foundation the United States Public Health Service and the Office of Naval Research More detailed reviews of these and other aspects of the biochemistry of visual systems have recently been published by Morton and Pitt (1957) Dartnall (1957) and Brindley (1960)

lies far over toward reduction (cf Bliss, 1951), and it can be driven in the oxidative direction only to the degree that retinene is removed as fast as formed

In the retina such "trapping" of the retinene is accomplished by opsin The combination of retinene with opsin is a spontaneous reaction, requiring neither enzyme nor a source of energy (Wald and Brown, 1950) The synthesis of visual pigment proceeds as a coupled reaction, in which the continuous removal of retinene to form the pigment promotes the continuous oxidation of vitamin A When, as in dark adaptation, all the opsin has been converted to visual pigment, the oxidation of vitamin A automatically ceases (Wald and Hubbard, 1950) Any other process in the organism that depended upon the oxidation of vitamin A to its aldehyde would require some similar trapping device

The gross arrangement of the four visual systems may therefore be expressed diagrammatically as follows



To make a visual pigment, it is not enough to have vitamin A or retinene, these molecules must have the right *shape* Each of them exists in a variety of configurations, *cis trans* or geometric isomers of one another (Fig 1) The most stable, and hence commonest, form is all *trans*, but this is valueless for making visual pigments To combine with opsin, a *cis* form of retinene is needed (Hubbard and Wald, 1952–1953)

Vitamin A possesses four double bonds in the hydrocarbon side chain, any one of which might be thought to occur in either *cis* or *trans* configuration There were good reasons, however, to expect that stable forms of this molecule would have *cis* linkages only at double bonds 9 or 13 A *cis* linkage at double bond 7 or 11 would encounter steric hindrance, the rotation through 180° that ordinarily accompanies the shift from a *trans* to a *cis* linkage could not be completed Either methyl groups would collide, as at double bond 7, or a methyl group would encounter a hydrogen, as at double bond 11 At such *hindered cis* linkages, the molecule would be not only bent, as always at a *cis* linkage, but twisted Since molecular planarity promotes conjugation (resonance), and since resonance promotes stability, a twisted molecule is expected to be relatively unstable For this reason it was expected that only the four unhindered isomers of vitamin A or retinene would be found in appreciable quantity all *trans*, 13 *cis* (neo a), 9 *cis* (iso a), and 9,13 *dicis* (iso b) (Fig 1)

It is a peculiar and significant fact, however, that all the visual pigments so far analyzed in this regard, including representatives from rods and cones, systems based upon vitamin A_1 and A_2, and vertebrate and inverte-

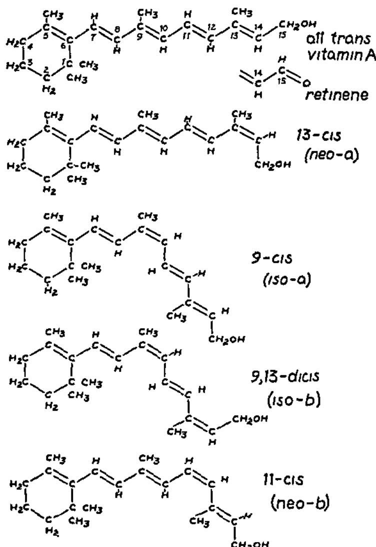


FIG 1 Structural formulas of geometric isomers of vitamin A and retinene The *cis* linkage can occur with relatively little steric hindrance only at double bonds 9 and 13 so that the four upper forms are relatively coplanar and represent the most stable configurations thermodynamically The 11 *cis* isomer is twisted as well as bent at the *cis* linkage owing to steric hindrance between the methyl group on carbon 13 and the hydrogen atom on carbon 10 This sterically hindered *cis* configuration of retinenes 1 and 2 constitutes the chromophores of all known visual pigments

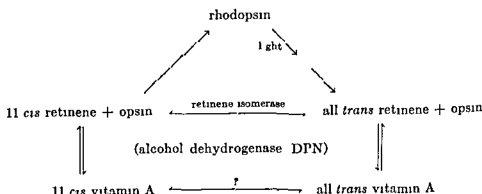
brate eyes are synthesized from and have as chromophore a sterically hindered *cis* isomer of retinene 1 or 2, the 11 *cis* isomer (neo b) *

* The structure of this isomer was established by synthesis (Oroshnik *et al* 1956)

This isomer, in spite of its steric hindrance, is surprisingly stable, once formed. It represents a higher energy state than the all *trans* configuration, and for this reason requires a special access of energy for its formation. In our experience this has been provided as light. Simple exposure of retinene to light causes isomerization to all possible configurations. In such a homopolar solvent as hexane, this ends in a steady state mixture, over 90% of which is the all *trans* isomer, but the same process carried out in a polar organic solvent such as ethanol ends in a steady state mixture in which only about half is all *trans*, and about 25% is 11 *cis*. That is, under these conditions, the hindered 11 *cis* isomer represents one of the most favored configurations (Brown and Wald, 1956).

One of the *unhindered cis* isomers of retinene 1 and 2 also combines with opsin to yield photosensitive pigments (Hubbard and Wald, 1952–1953). *This is the 9 cis isomer (iso a), the one which most resembles the 11 cis* isomer in shape. The pigments that result have properties very much like those of the visual pigments, though their λ_{max} in all cases lies at shorter wavelengths. We call them the iso pigments: isorhodopsin (λ_{max} 487 mμ), isoiodopsin (515 mμ), isoporphyropsin (507 mμ), and isocyanopsin (575 mμ). None of the iso pigments has yet been identified in a retina. We must regard them for the present as artifacts. Since however the 9 *cis* isomer of vitamin A occurs in liver and is transported by the blood, the retina must have some means of excluding it from forming iso pigments (P. S. Brown, unpublished observations, Brown *et al.*, 1959).

Whereas specific *cis* isomers of the retinenes and vitamins A are required to synthesize the visual pigments and the iso pigments, the bleaching of the latter by light yields invariably as main product the all *trans* isomer (Hubbard and Wald, 1952–1953). To resynthesize a visual pigment, this must be reisomerized to the 11 *cis* configuration. For this reason, a cycle of *cis trans* isomerization of the retinenes or the corresponding vitamins A is an intrinsic component of every known visual system. In the rhodopsin system this takes the form



Precisely similar diagrams may be written for each of the other vertebrate systems, in which vitamin A is replaced by A (porphyropsin Wald, Brown and Smith, cited in Wald, 1953), or rod by cone opsin (iodopsin, cyanopsin Wald *et al*, 1954–1955, 1953)

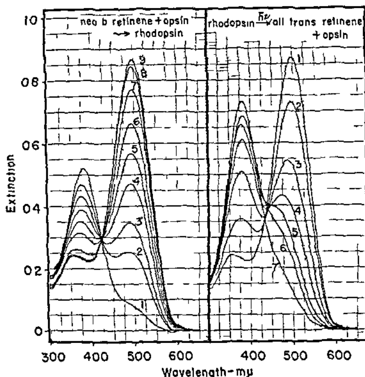


FIG 2 Synthesis and bleaching of rhodopsin in solution 22 5 C pH 7 *Left* a mixture of 11 *cis* retinene and cattle opsin are incubated in the dark and absorption spectra recorded (*1*) at 0 3 (*2*) at 2 5 (*3*) at 5 (*4*) at 10 (*5*) at 18 (*6*) at 30 (*7*) at 60 (*8*) at 120 and (*9*) at 180 minutes The absorption band of 11 *cis* retinene (λ_m = 380 mμ) falls regularly whereas that of rhodopsin (λ_m = 498 mμ) rises *Right* the rhodopsin formed at the left (1) is now exposed to light of wavelengths longer than 550 mμ for various intervals and the absorption spectra are recorded immediately The total irradiations are (*2*) 5 (*3*) 10 (*4*) 15 (*5*) 30 and (*6*) 120 seconds The residue was exposed for 45 seconds longer to light of wavelengths longer than 440 mμ (*7*) From Wald and Brown (1956)

Figure 2 shows one passage of rhodopsin through the isomerization cycle (Wald and Brown, 1956) On the left, 11 *cis* retinene and cattle opsin combine to form rhodopsin On the right, this rhodopsin is bleached by light to a mixture of all *trans* retinene and opsin The retinene that emerges from bleaching has about 1 5 times the extinction of that which entered the synthesis That is because all *trans* retinene has about 1 7 times the molar extinction of the 11 *cis* isomer

The mechanisms by which the eye converts all *trans* retinene or vitamin A to the 11 *cis* isomer for the synthesis of visual pigments are not as yet wholly understood This isomerization must proceed rapidly enough to account for the persistence of vision in bright light, in this condition the visual pigments may be bleached almost as soon as formed, and vision depends on their continuous resynthesis

As already noted, simple exposure to light isomerizes retinene to mixtures of all possible isomers, eventually in some steady state proportion This cannot be an important source of the 11 *cis* isomer *in vivo*, however, because retinene appears in the visual process only as a transient intermediate which never accumulates, and because the isomerization is accomplished only by such short wavelength light (mainly violet and ultraviolet) as retinene can absorb Relatively little of this radiation penetrates to the retina

The eye tissues contain an enzyme, retinene isomerase, which catalyzes specifically the interconversion of all *trans* and 11 *cis* retinene (Hubbard, 1955–1956) In dim light a mixture of these isomers results, which contains about 32% of the 11 *cis* isomer In the dark, however, the same enzyme catalyzes the approach to what is probably the thermodynamic equilibrium, in which 95% of the mixture is all *trans* This enzyme system therefore might be of some help in the light, but its effects could persist through a dark interval only if means existed for removing 11 *cis* retinene from its influence

It is clear, however, that the eye also possesses means for converting the all *trans* isomer to 11 *cis* in darkness, working in this case presumably with vitamin A, though perhaps in some bound or activated condition (Hubbard and Colman, 1959, Dowling, 1960) We do not yet know the mechanism of this process It poses an interesting problem for this is an energy demanding (endergonic) change and requires activation or coupling with another, exergonic reaction The process can on occasion be extraordinarily effective, achieving in certain invertebrate eyes, as noted below, a virtually exclusive accumulation of 11 *cis* vitamin A

In any case, the formation of the 11 *cis* isomer in appreciable quantities seems to be restricted, so far as we know, to the eye tissues We have not yet succeeded in identifying this isomer in liver, kidney, or blood (P S Brown, unpublished observations)

More or less similar relationships to those described above involve the visual systems of invertebrates In every known case, these are derived from vitamin A_1, and the chromophores of the visual pigments are retinene$_1$ (Wald, 1943 1960) * In all cases which have been sufficiently analyzed,

* In addition to a very limited survey of the vitamins A of invertebrate eyes by Wald (1943) individual investigations of invertebrate visual pigments now comprise squid (Wald 1943 St George and Wald 1949 Hubbard and St George 1957–1958)

furthermore the chromophore has the 11 *cis* configuration (Wald and Burg 1956–1957, Wald and Brown, 1956–1957, Hubbard and St George, 1957–1958) We have not yet met an active alcohol dehydrogenase system in any invertebrate eye, such as might reduce the retinene formed by light to vitamin A rapidly enough to play a direct role in the visual cycle In all such eyes yet examined, the action of light on the visual pigments ends at most in the production of retinene

On the other hand, some invertebrates (squid, lobster, euphausiids) present us with the peculiar condition of restricting vitamin A almost wholly to the eye More than 90% of the total vitamin A which such animals possess may be found in the eye and frequently none at all can be identified in the remaining tissues (Wald, 1943, Fisher *et al*, 1952, 1955) What is more remarkable, within the limits of accuracy of the measurements, all the vitamin A stored as the free substance in the eye of the lobster and of a euphausiid crustacean, *Meganyctiphanes*, was found to be in the 11 *cis* configuration (Wald and Burg 1956–1957, Wald and Brown, 1956–1957) How these organisms succeed in producing and maintaining this condition against a highly adverse thermodynamic gradient poses an interesting problem

The geometrical specificity displayed by the visual pigments in synthesis and bleaching is the central phenomenon in the visual process So far as we know *the only thing that light does in vision is to isomerize retinene*

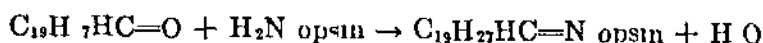
Retinene by virtue of its end group, exhibits many of the usual aldehyde reactions (Ball *et al* 1948) If, however, its union with opsin involved only the aldehyde group, the geometry of the hydrocarbon chain would be irrelevant Actually it is decisive Not only is the combination with opsin restricted to 9 and 11 *cis* retinene, but each of these isomers yields as we have seen, a different product Incidentally this last point shows that retinene maintains its specific *cis* configuration while serving as the chromophore of a visual pigment or iso pigment If in becoming chromophores the *cis* retinenes were isomerized to all *trans*, only one product could result

It seems clear that the point of this geometrical specificity in forming photosensitive pigments is that of all the retinene isomers, only the 11 *cis*, and to a lesser degree the 9 *cis* configuration *fit* the surface of the opsin in the region of attachment, so permitting intimate interactions between the retinene and protein that account for the pigment's color, stability, and photosensitivity On absorbing a quantum of light, the retinene is isomerized to all *trans* Now it no longer *fits* the opsin and hence is dissociated from it This in short is the mechanism of bleaching

There is reasonably good evidence that the covalent attachment of

octopus and cuttlefish (Brown and Brown 1958) euphausiids (Kampa 1955) lobster (Wald and Hubbard 1957) bee (Goldsmith 1958) and *Limulus* (Hubbard and Wald 1960)

retinene to opsin is through a Schiff base linkage formed by condensin aldehyde group of retinene with an amino group of opsin (Ball *et al*, Collins, 1953, Morton and Pitt, 1955)



$$C_{19}H_{27}HC{=}O + H_2N\ \text{opsin} \rightarrow C_{19}H_{27}HC{=}N\ \text{opsin} + H_2O$$

One of the main problems in formulating the further interactions bet retinene and opsin is to account for their large effect upon the absor spectrum The λ_{max} of 11 *cis* retinene is at about 380 mμ, the attach to opsin shifts this about 120 mμ toward the red in rhodopsin, and a 180 mμ in iodopsin

What is said further about this problem is still largely hypothe (Hubbard, 1958) Such a Schiff base linkage as formulated above wou itself shift the absorption spectrum of 11 *cis* retinene toward *shorter* w lengths, to about 360 mμ The attachment of a hydrogen ion (protona $C_{19}H_{27}HC{=}NH^+$ opsin), however, would shift the spectrum to about mμ, owing to the introduction of an electric charge which oscillates thr the *conjugated system of retinene* *To protonate a Schiff base in sin* instances requires a high acidity (Morton and Pitt, 1955) Here we r assume not only that it happens in neutral solution, but that the pigr can hold the proton even in mildly alkaline solution, for none of these ments changes color with pH, and rhodopsin is reasonably stable up tc 10 (Radding and Wald, 1955–1956b) To obtain the considerably fur shift of spectrum toward the red found in the visual pigments, we ass special interactions with opsin that depend on closeness of fit They i include the presence in the area of linkage of a negatively charged gr on opsin, which by drawing up into the conjugated chain the posi charge brought in by H^+, might further promote resonance This incre resonance might also account for the remarkable stability of the Schiff l linkage (such linkages ordinarily hydrolyze rapidly in neutral solution), for its extraordinary capacity, already noted, to hold a hydrogen ion

These problems are as yet far from settled I think however that principle is clear, that closeness of fit between retinene and opsin acco for the synthesis and many of the principal properties of the visual ments and iso pigments It is this factor that singles out for the synth of photopigments the 9 and 11 *cis* isomers, which most resemble each ot in shape The 11 *cis* isomer *fits* the opsins better than the 9 *cis* isomer, this reason the iso pigments as a class have shorter λ_{max} and are also stable than the visual pigments

Both rhodopsin and isorhodopsin are bleached by heat as well as light Just as when bleached by light, the immediate cause of bleachin the loss of fit between retinene and opsin, but heat brings this about changing the configuration of opsin through denaturation The retinen

not directly affected It emerges from rhodopsin mainly in the 11 *cis* configuration, and from isorhodopsin as the 9 *cis* isomer (Hubbard, 1958–1959)

On the other hand, isorhodopsin is bleached by heat at a somewhat lower temperature, and with a slightly lower Arrhenius energy of activation than rhodopsin one evidence that 9 *cis* retinene fits opsin less well, and hence yields less stable pigments (Hubbard, 1958–1959)

When visual pigments are bleached by light, however, the immediate cause of bleaching is the loss of fit owing to isomerization of retinene Such photobleaching proceeds in a sequence of stages, including the highly colored intermediates lumi and metarhodopsin, in which retinene remains

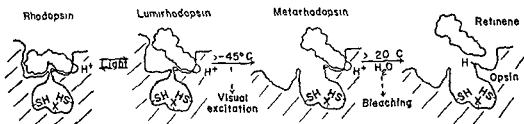


FIG 3 The action of light on rhodopsin The absorption of light by rhodopsin isomerizes its 11 *cis* chromophore to the all *trans* configuration yielding as first product the all *tran* chromoprotein lumirhodopsin This labilizes the protein opsin which rearranges to a new configuration yielding a second all *trans* chromoprotein metarhodopsin This second process exposes reactive groups on opsin (two —SH groups (Wald and Brown 1951–1952) and one H^+ binding group (Radding and Wald 1955–1956a)) and may be responsible for triggering visual excitation Vertebrate metarhodopsins are unstable and above about −15 C hydrolyze to all *trans* retinene and opsin the process that corresponds to bleaching Modified from Hubbard and Kropf (1958 1959)

attached is chromophore to opsin, and ending with the actual bleaching that accompanies the dissociation of retinene from opsin (Wald *et al* 1950) In the past we have used the terms lumi and *metarhodopsin for what we* now recognize to have been mixtures of pigments We now define these terms to mean the intermediate pigments that possess all *trans retinene as* chromophore as in the following account (see Figs 3 and 4) (Hubbard and Kropf, 1958, 1959 Kropf and Hubbard 1958)

Light initiates bleaching by isomerizing the 11 *cis* chromophore of a visual pigment to all *trans* The product, itself highly colored, is *the lumi* pigment (lumirhodopsin, etc) It is composed of all *trans* retinene attached specifically at the chromophoric site to unaltered opsin Such lumi pigments are stable in glycerol water mixtures below −50° C

On warming to −20° to −15° C a lumi pigment changes in the dark to a corresponding meta pigment (metarhodopsin, etc) This is still highly

colored, generally red orange, and has all *trans* retinene as chromophore The change from the lumi- to the meta pigment seems to involve an alteration in the configuration of opsin, inhibited at the lower temperature in the highly viscous glycerol water mixture

The meta pigments are stable below $-20°$ C, but on warming to higher temperatures, and in the presence of water, they hydrolyze in the dark to mixtures of all *trans* retinene and opsin, the final products of bleaching

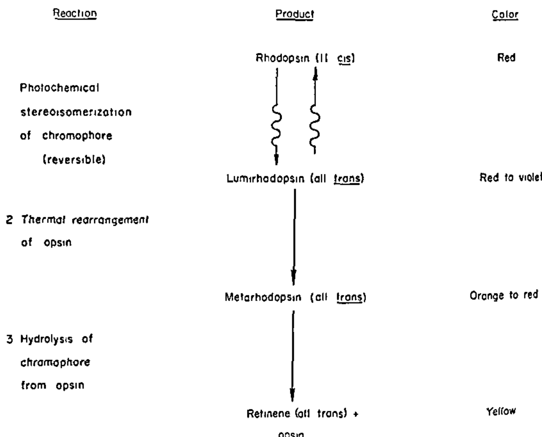


FIG 4 Summary of the stages in bleaching rhodopsin with light *Adapted from* Hubbard and Kropf (1958, 1959)

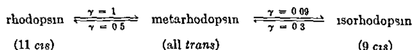
This sequence of changes is summarized in Fig 4 Light is involved in only the first step, the isomerization of 11 *cis* retinene to all *trans*, converting a visual pigment to the corresponding lumi pigment The remainder of the sequence, and indeed all the other processes in visual excitation, consist of ordinary thermal ("dark") reactions

Visual excitation must depend upon the initial steps in this sequence, the formation of the lumi or at most the meta pigments (Wald *et al*, 1950) The hydrolysis to retinene and opsin is much too slow a process to account for excitation Indeed, in a number of invertebrate eyes (squid, lobster)

frank bleaching hardly occurs under physiological conditions, the action of light on the visual pigments ending with the production of metarhodopsin (cf Wald 1960)

We have already mentioned that simple exposure to light isomerizes retinene eventually to a steady state mixture of all possible isomers, and that in a polar solvent about 25% of this steady state mixture is 11 *cis*, about 25% other *cis* isomers, and about half all *trans* Similar isomerizations can occur with retinene attached as chromophore to opsin If rhodopsin, for example, is irradiated at about −60° C in a glycerol water mixture, the first quantum absorbed by the molecule isomerizes 11 *cis* retinene to all *trans*, yielding lumirhodopsin, but the absorption of further quanta at this temperature continues to isomerize retinene, ending in such a steady state mixture of isomers as just described Since, however, in this case the retinene remains attached to unaltered opsin at the specific chromophoric site, the fraction of this mixture which is 11 *cis* is now again rhodopsin—rhodopsin made from lumirhodopsin through reisomerization of the retinene by light Another fraction, which is 9 *cis*, is now isorhodopsin, also made so by light The remainder of the retinene is in configurations that do not serve as the chromophores of photopigments all *trans*, 13 *cis*, etc , and this is the fraction—about half the total—that bleaches to retinene and opsin on warming in the dark A mixture of rhodopsin and isorhodopsin constitutes the remaining half of the product

A similar steady state is instituted when metarhodopsin, stabilized by holding below −20 C , is irradiated with visible light The form of this steady state is shown in the following diagram, together with estimates of the quantum efficiencies of the component photoreactions (Hubbard and Kropf, 1958, 1959 Kropf and Hubbard 1958) In this diagram the quantum yield of the isomerization from the 11 *cis* to the all *trans* chromophore is set arbitrarily at 1, and the others are rated accordingly



(11 *cis*) (all *trans*) (9 *cis*)

When it was first found that about half the product of bleaching rhodopsin in this way is still in the form of photosensitive pigment it was thought that the latter must result from some "dark" reversion process We now realize that, on the contrary, this residue of rhodopsin and isorhodopsin is formed as described, by the light reaction Similarly, it is sometimes stated on the basis of the observations just described, that the quantum efficiency of bleaching is only about one half In fact the efficiency of the first quanta absorbed—which convert the visual pigment to the lumi pigment—may be 1 When the over all efficiency of bleaching is less than 1 it is largely

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Vitamin A and Proteins

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I Introduction

Our strongest evidence that vitamin A plays a special role in protein metabolism relates to the synthesis of the mucoproteins, which will be discussed by Dr Wolf The possible influence of vitamin A on the balance between SH and SS groups (Redfearn and Strangeways, 1957) also seems relevant to this subject Further examples of association between vitamin A and protein are found in the formation of certain vitamin A protein complexes, such as the familiar rhodopsin and the albumin complex which carries vitamin A in the blood plasma (Dzialoszynski *et al*, 1945)

Apart from these specialized functions and compounds, however, there is little or no indication that vitamin A has any specific influence on the general metabolism of protein throughout the body Thus vitamin A has no dramatic role to parallel that shown by vitamin E in the prevention of massive liver necrosis in animals deprived of protein At the most experiments have shown that there may be some interplay between the levels of the protein and vitamin A intakes in their combined effect on the condition of the animal

For convenience we may divide the treatment of our topic into two parts First we may discuss the influence of variations of the protein intake on the condition of animals completely deprived of vitamin A, or receiving only low doses Secondly we may change our viewpoint and consider the effect of varying the dose of vitamin A when the intake of protein is grossly inadequate Some experimental findings, which my colleagues and I obtained long ago in work that has not hitherto been described in detail, may be of interest in regard to this second line of approach

II THE EFFECT OF THE ADEQUACY OF THE PROTEIN INTAKE ON THE VITAMIN A STATUS

In his classic observations on xerophthalmia in Danish infants, Bloch (1921) drew attention to the importance of various stresses, including intercurrent diseases not directly related to avitaminosis A, in precipitating the development of xerophthalmia One form of stress appeared to be a diet containing excessive amounts of carbohydrate, and photographs of the afflicted infants suggest that they were suffering from the condition now known as kwashiorkor On this evidence, therefore, deficiency of protein may aggravate the injuries caused by a simultaneous lack of vitamin A

More recent experiments on rats by McLaren (1959) have given different results Thus in aimals of which the growth rates were limited by inadequate allowances of protein, the time of the appearance of xerophthalmia was delayed It was also found that male rats succumbed more rapidly than females when deprived of vitamin A, provided that their allowances of protein were sufficient for them to display their natural capacity for more rapid growth

It seems probable therefore that the protein allowance can affect the vitamin A status in at least two ways First, a good allowance of protein promotes rapid growth, and so requires a high rate of expenditure of vitamin A, with a rapid depletion of the preliminary reserves Secondly, a very low intake of protein can lead to fatty infiltration of the liver, and deterioration of the powers of digestion and absorption by the intestinal tract These changes will reduce the efficiency of utilization of any small amounts of vitamin A and provitamins that may be present in the deficient diet, and so accelerate the appearance of the signs of deficiency With these two influences capable of acting in opposite directions, it is obvious that the effect of protein deficiency on the vitamin A status will vary in different clinical or experimental circumstances

The effect of graded levels of protein intake on the growth responses of rats given small graded doses of vitamin A was studied by Dye and her colleagues (1945) At levels of dosing of 1, 3, or 6 I U of vitamin A daily, growth was always slightly more rapid when the diet contained 18% of protein than when it contained either 9 or 36% Under the conditions of these experiments, however, the adequacy of the dose of vitamin A had much more influence on growth than had the variations in the protein intake

Several investigations have been made on the influence of the protein intake on the storage of vitamin A in the liver Basu and De (1941) reported that when rats were given a diet containing 18% of protein, in conjunction with daily doses of 100 I U of vitamin A, they stored much more vitamin

A in their livers than when the same dose of vitamin A was given, but with a diet containing only 8% of protein Baumann and his colleagues (1942) also found that a low protein intake tended to reduce the storage of vitamin A in rats According to Moore (1940), partial deficiency of protein sometimes, but not always, caused the storage of vitamin A to be slightly decreased in the rat We may presume that the influence of the protein allowance on the storage of vitamin A will resemble its influence on the appearance of the signs of deficiency and will be capable of varying in its direction under different experimental conditions Doubtless experiments could be designed, with animals capable of rapid growth in which an adequate protein allowance would cause a more rapid depletion of the liver reserves of vitamin A than that occurring in stunted animals deprived of protein

III The Effect of Liberal Doses of Vitamin A on Resistance to Protein Deficiency

Two experiments on this topic, both with rats as the experimental animal, will be described

Experiment 1 A short account of this experiment has already been given (Moore *et al*, 1952) Piebald male rats weighing 75–88 gm, were first given our standard diet deficient in vitamin A It contained casein, 20, sucrose, 60, arachis oil, 15, dried yeast, 10, and minerals 5 parts Adequate doses of vitamins D, E, and K were provided After this diet had been received for about 3 months the growth of the animals slackened and became irregular The incisor teeth of most of them showed loss, or mottling, of the normal brown pigment They were therefore judged to be deficient in vitamin A

At this point the diet was changed so as to be both deficient in vitamin A and low in protein It now contained sugar 75, lard 10 dried yeast,10, and minerals, 5% with the usual supplements of vitamins D, E, and K Four of the rats (Group 1) were dosed with 5000 I U of vitamin A daily for 3 days, and subsequently with 1000 I U weekly Another four rats (Group 2) were dosed only at the marginal level of 28 I U weekly

Curves giving the mean body weights for each group, over the next 3 months, are shown in Fig 1 It will be seen that the rats of Group 1, liberally dosed with vitamin A, at first increased in weight in spite of the deprivation of protein Later there was a slow decline, but even at the end of the period of observation the mean body weight remained above the level found when deprivation of protein was started In contrast the weights of the rats in Group 2 given marginal doses of vitamin A, steadily declined and after about 7 weeks of protein deprivation one of the animals died The remaining three rats were eventually given a diet adequate in protein (casein, 25, sucrose,

50, lard, 10, dried yeast, 10, and minerals, 5%), without any change in their allowance of vitamin A It will be seen from Fig 1 that there was a prompt increase in body weight Under the conditions of this experiment, therefore, a liberal intake of vitamin A was to some degree beneficial in enabling rats to resist loss of body weight during deprivation of protein

Experiment 2 Piebald male rats, of body weights 39–56 gm , were first kept for about 7 weeks on a diet deficient in vitamin A It contained casein, 25, sucrose, 50, lard, 10, dried yeast, 10, and minerals, 5% Vitamins D,

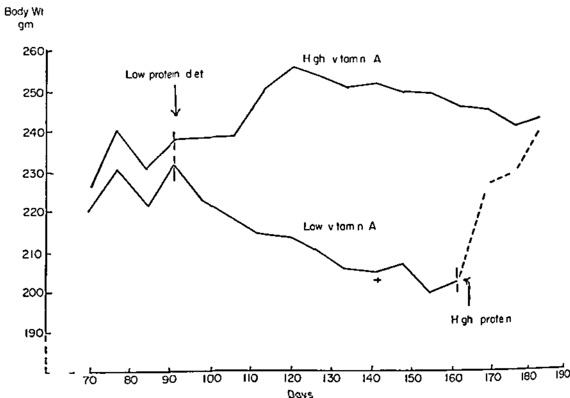


FIG 1 Mean body weights of rats given a diet low in protein and with high (Group 1) or low (Group 2) doses of vitamin A

E, and K were provided The animals were then divided into groups Some continued to receive the diet containing casein, but for others the casein was replaced by additional sucrose With each level of protein, doses of vitamin A were given at three levels (see Table I)

Figure 2 shows curves for the mean body weights in the various groups over the next 10 weeks In the groups receiving the diets low in protein, maintenance of body weight was better with 40 I U of vitamin A daily than with 4 I U , and was better still with 320 I U In agreement with the finding in the first experiment, therefore, liberal doses of vitamin A helped the rats to maintain their body weights when their diet was deficient in protein In the groups that received adequate amounts of protein, growth

TABLE I

EXPERIMENT 2 ARRANGEMENT OF GROUPS

Group No	Number of rats	% Casein	Daily dose of vitamin A (I U)
1	6	25	4
2	5	25	40
3	4	25	320
4	10	0	4
5	10	0	40
6	10	0	320



FIG 2 Mean body weights of rats given diets high or low in protein and with graded daily doses of vitamin A

was observed at all three levels of dosage with vitamin A, but was much more rapid with 40 or 320 I U daily than with only 4 I U daily

In this experiment some of the rats were killed, after longer periods on the same diets, for the estimation of the amounts of vitamin A and fat in their livers For this purpose the livers of rats of the same group were pooled

The results are given in Table II It will be seen that in two of the groups given an adequate allowance of casein (Groups 1 and 3) the concentration of vitamin A was greater than in the corresponding groups which were deprived of protein This finding agrees with the general experience, gained in investigations described in the preceding section, that deficiency of protein tends to lower the vitamin A reserves The liberal doses of vitamin A obviously did not prevent the fatty infiltration of the liver which is to be expected in protein deficiency

TABLE II

MEAN LIVER WEIGHTS, AND CONCENTRATIONS OF VITAMIN A AND FAT IN THE LIVERS OF RATS GIVEN DIFFERENT ALLOWANCES OF VITAMIN A IN CONJUNCTION WITH DIETS ADEQUATE OR DEFICIENT IN PROTEIN

Group No	Number of rats	% Casein in diet	Daily dose of vitamin A (I U)	Mean weight of liver	Vitamin A (I U /gm liver)	% Fat in liver
1	5	25	4	10 3	30	5 2
2	4	25	40	11 4	77	5 0
3	4	25	320	12 8	2200	6 4
4	5	0	4	10 9	5	35 0
5	5	0	40	13 4	72	33 5
6	5	0	320	13 3	1300	30 0

IV CONCLUSIONS

As already hinted the results of the experiments just described, taken in conjunction with those of the earlier work which has been reviewed, do not point to any close relationship between vitamin A and the general metabolism of protein There is evidence, however, that the intakes of vitamin A and protein may to some extent interact in their influence on the condition and nutritional status of the animal The effects of this interaction may be seen in the time of appearance of the signs of deficiency, in the stores of vitamin A present in the liver, or in growth or the maintenance of body weight

New evidence that growth and the maintenance of body weight may be affected simultaneously by the intakes of both vitamin A and protein is supplied by the experiments just described in Section III It has sometimes been taken as an axiom by nutritionists that the health of an animal, and more particularly the rate of growth, will be limited by that one nutrient which is least adequately supplied by the diet Doubtless there are some circumstances, particularly in carefully planned experiments designed to produce a specific deficiency, when this axiom is substantially true It

would seem rash, however, to assume that a combined deficiency of two or more nutrients can impose no more strain on the animal than could deficiency of a single nutrient In the experiments described, the rats did better when they were deficient only in protein than when they were also inadequately supplied with vitamin A

Another interesting point was the benefit which appeared to be gained from giving liberal doses of vitamin A, as compared with marginal doses which seemed adequate to prevent danger of acute deficiency Research on the possible value of liberal doses of vitamin A in increasing the resistance to forms of stress other than protein deficiency might be instructive The work of Meunier and his colleagues (1949 1950) on the influence of vitamin A on detoxicating mechanisms has already given a start in this direction

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Vitamin A and Mucopolysaccharide Biosynthesis

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I INTRODUCTION

In a search for a biochemical function of vitamin A in metabolism, when looking over the existing literature on the effects of vitamin A deficiency or excess one is immediately struck by the importance of the influence of vitamin A on mucus formation and on mucosal tissue Moore (1957a), in his monumental work on vitamin A, states 'If we consider the role of vitamin A on the widest possible basis therefore, we may say that it is necessary for the formation of large molecules containing glucosamine" Such large molecules are the mucopolysaccharides which in addition, contain galactosamine, glucuronic acid, sulfate, and in certain cases also sialic acid, mannose and fucose Any hypothesis of a systemic function of vitamin A therefore, has to take into account its effect on the biosynthesis of the mucopolysaccharides These occur in various forms, and with various functions in almost all tissues of the mammalian organism, but principally, and in largest quantities in two locations in the mucus secreted by mucous epithelium, and in the extracellular matrix of cartilage mainly as chondroitin sulfate Attention will be focused in this review on the influence of vitamin A on these two tissues

II. *In Vivo* Observations

1. *Vitamin A Deficiency*

Vitamin A deficiency shows its first manifestation in a disruption and atrophy of all those epithelial tissues that function in mucus secretion in the respiratory, intestinal, and urinary tracts and in the genital system (Wolbach and Howe, 1925). A decrease in the number and activity of mucus secreting cells of the gastrointestinal tract occurs (Manville, 1937). Ultimately, a new type of epithelium replaces the mucus secreting tissue. It becomes keratinized and similar to epidermis. The effect of the deficiency on the other important mucopolysaccharide of the mammal, the chondroitin sulfate of cartilage, is quite different: increased activity of osteoblasts, periosteal overgrowth with the formation of larger, soft bones (Mellanby, 1950). The effect of the deficiency on mucus formation in the genital system, especially vaginal epithelium, is particularly instructive, as here there are cells that at one time of the estrous cycle become mucus secreting, at another time keratinized. The whole picture is well summarized by Moore (1957b): the vaginal epithelium, even in the absence of estrogen, becomes keratinized in vitamin A deficiency. In the absence of estrogen, but with excess vitamin A, it is transformed into mucus secreting epithelium, a similar change being caused by progesterone, but only in the presence of normal levels of vitamin A. Estrogen, even in the presence of vitamin A, stimulates keratinization. The conclusion from these observations is that the normal state of this epithelium is to be keratinized, vitamin A stimulates the transformation to mucous epithelium. One could then further speculate that estrogen inhibits this action of vitamin A, whereas progesterone overcomes the inhibition.

Since esterified sulfate represents an important part of the mucopolysaccharide molecule, a number of investigations have considered the effect of vitamin A deficiency on sulfate incorporation into cartilage. Dziewiatkowsky (1954) isolated chondroitin sulfate, the principal substance, apart from collagen, of cartilage matrix, after injection of labeled sulfate into vitamin A deficient, normal, and vitamin A treated deficient rats. The level of labeled sulfate was higher in the normal and vitamin A treated deficient rats than in the deficient. This author also found increased breakdown as well as synthesis of chondroitin sulfate in the vitamin A treated animals. Somewhat different results were obtained by Frape *et al.* (1959) working with vitamin A deficient pigs. Here, vitamin A treatment of the deficient animals caused *decreased* sulfate uptake into cartilage as the primary response (Table I).

2 Hypervitaminosis A

Vitamin A is distinct from other vitamins in that it acts when administered in excess the effect being generally the reverse of that obtained in deficiency Maturation of cartilage cells is accelerated, cartilage, as well

TABLE I

S^{35} ACTIVITY IN TISSUES FROM PIGS ON VARIOUS DIETARY VITAMIN A LEVELS

Vitamin A added per pound of feed I U	5th Rib costochondral junction[b]	Ear cartilage[b]	Lung
		counts/min /gm	of fresh tissue
0	87581	21213	7053
55	42622	26764	3442
208	48601	5902	4033
790	32554	10811	1731
1540	46789	12812	1346
3000	54014	17082	1761
11393	53221	22238	2087

From Frape et al (1959)
[b] Values are average of two samples
Values are average of three samples
[d] Counting rates are corrected for background decay and extrapolated to zero thickness of precipitate

TABLE II

EFFECT OF HYPERVITAMINOSIS A ON SERUM CONCENTRATIONS OF MUCOPROTEIN

Group[b]	Number	Vitamin A dose	Age at killing (days)	Serum mucoprotein (mg tyrosine %)
An	2	0	39–40	5 8(2) ± 0 05
Ah	2	0	39–40	6 3(2) ± 0 70
Cn	3	1000	39–40	10 3(2) ± 0 05
Ch	4	1000	39–46	16 3(4) ± 3 74

From Cohen et al (1955)
[b] n Refers to the group of animals on the diet normal in calcium h refers to those on a diet high in calcium content
The figures in parenthesis refer to the number of animals or chemical determinations in the group

as bone, is rapidly absorbed, resulting in spontaneous fractures (Wolbach, 1947) Normal vaginal and uterine epithelium is replaced by noncornifying epithelium (Hedenberg, 1954) Mucoprotein levels in blood are greatly increased (Cohen et al 1955) (Table II)

Recently Thomas et al (1960) showed that, in the intact animal, hypervitaminosis A resulted in a depletion of cartilage matrix due to loss of cartilage chondromucoprotein, and a consequent rise in serum chondroitin

sulfate It should be noted that this is the reverse of the effect of vita
excess on mucous tissue, where the hypervitaminosis results in a *sti
tion* of mucus secretion, reflecting, no doubt, a different mode of act
vitamin A on mucopolysaccharide of connective tissue and of mucoi
sue

III Observations on Tissue Culture

All the work reported here on the effect of vitamin A on tissue c
has been carried out by H B Fell and her collaborators (reviewed by
1953) The objective was to determine whether the effects of vitar
deficiency and hypervitaminosis A described in the preceding sections
indirect and mediated through an endocrine organ, or directly on th
sue First, in their work on explants of undifferentiated chick en
bones, cultured in a medium with excess vitamin A, Fell and Mel
(1952) found the bone to turn soft and gelatinous, growth to dim
and fractures to occur The cartilage matrix ceased to stain with basc
dyes, metachromasia, that is, the response of the stain toluidine bl
the acid groups of chondroitin sulfate, disappeared It was thought
whereas there occurred dissolution of the chondroitin sulfate, the col
part of the matrix remained intact With fully developed fetal n
bones, the situation was similar—cartilage matrix and bone were abs
and disappeared The vitamin was present in a concentration simil
that found in the blood There was then no doubt that the action of
min A was direct, though only on growing cells

In their second set of experiments, Fell and Mellanby (1953) cult
undifferentiated embryonic chick ectoderm This normally develo
keratinizing layer over squamous epithelium Cultured in excess vitam
it formed instead ciliated columnar cells with secretory vacuoles, act
secreting mucus, closely similar to nasal epithelium When returne
normal medium, mucus secretion continued, but no new mucous
differentiated and ultimately a new layer of squamous cells was fo
which eventually keratinized

The changes described are all basically those seen in the whole an
though greatly exaggerated Similar experiments with skin from mam
or birds showed less pronounced transformations than with embry
skin

These important results demonstrate that the action of vitamin
twofold (1) it causes dissolution of chondroitin sulfate of cartilage ma
(2) it affects epithelial basal cells in such a manner as to make them
ferentiate into mucus secreting cells, when they would normally
keratinizing cells The conclusion is inescapable that vitamin A ha
action akin to that of a hormone in determining the course of differe
tion of the basal cells of epithelium

It is of interest to note that the effect of excess vitamin A in tissue culture on the chondroitin sulfate of cartilage leads to dissolution and disappearance, whereas on the related mucoid substances of mucous epithelium, it causes sulfate uptake and synthesis (Fell *et al*, 1954) Though the properties and identity of the mucopolysaccharides of mucus are not completely established (see Section IV, 1), they are certainly different from chondroitin sulfate of cartilage It is possible therefore to conclude that excess vitamin A increases the mucus type and decreases the connective tissue type of mucopolysaccharide The reverse, a decrease in mucus and increase in connective tissue mucopolysaccharide, along with keratinization occurs in deficiency These generalizations help to interpret the *in vivo* data discussed in Section II decrease in mucus secretion and periosteal overgrowth in vitamin A deficiency, increased mucus secretion, increased breakdown of chondroitin sulfate from cartilage, less sulfate uptake into cartilage, when vitamin A is administered

IV *In Vitro* Observations

1 *Chemistry and Biochemistry of Mucopolysaccharides*

Many excellent reviews exist of recent work in the chemistry of the mucopolysaccharides (American Society of Biological Chemists Symposium, 1958 Springer 1958) However for a clearer understanding of the subsequent discussion a brief summary of the chemistry and biochemistry of those mucopolysaccharides on which vitamin A has an influence is relevant

Three different chondroitin sulfates have been isolated (Meyer, 1958) Chondroitin sulfate A, found in cartilage, bone cornea, and aorta, is a polymer of D glucuronic acid and sulfated *N* acetylgalactosamine, with *N* acetylchondrosine sulfate (I) as the repeating unit The two subunits are linked in a β 1,3 glucuronidic link and a β 1,4 hexosaminidic link, with sulfate esterified on position 4 of the acetylgalactosamine

Chondroitin sulfate B (repeating unit, II), found in skin tendon, and aorta is identical with A except for epimerization at C 5 of the glucuronic acid, leading to L iduronic acid

In chondroitin sulfate C (repeating unit, III) obtained from cartilage, umbilical cord, tendon and sclera, the sulfate group is shifted to position 6 of the acetylgalactosamine moiety

Heparin is defined by Jeanloz (1958) as "dextrorotatory sulfated polysaccharides composed of D glucosamine and D glucuronic acid" of various degrees of sulfation The sulfate in these compounds is linked to the amino group in a sulfamic link, although compounds related to heparin, containing glucosamine and glucuronic acid with *N* acetyl and *O* sulfate glucosamine have been found (heparitin sulfate)

fully formed polymer Recent evidence (Suzuki and Strominger, 19 would favor the second possibility Sulfate, before entering this reacti is activated by reaction with adenosine triphosphate to give adenos 5' phosphosulfate, which is further phosphorylated to 3' phosphoade sine 5' phosphosulfate ("active sulfate") (Robbins and Lipmann, 1957)

2 *Studies with Colon Homogenates and Vitamin A*

From *in vivo* observations and tissue culture data, indications w strongly suggestive of an involvement of vitamin A in a controlling s in mucopolysaccharide biosynthesis, in particular in stimulation of su synthesis in a mucus forming system For this purpose, Wolf and Var dani (1960) developed an *in vitro* system, consisting of rat colon segme

TABLE III
INCORPORATION OF GLUCOSE U C^{14} INTO HEXOSAMINES OF MUCOPOLYSACCHARI SYNTHESIZED BY COLON HOMOGENATES

	c p m
Total C^{14} mucopolysaccharides isolated	15 200
Total C^{14} hexosamine obtained therefrom	6 232
Specific activity of hexosamine	
	c p m /mmole
After addition of carrier (54 μmoles)	5 000
After 2nd crystallization of osazone	6 800
After 3rd crystallization of osazone	6 750

[a] The complete system consisted of 10 μmoles ATP 6 μmoles of $MgCl_2$ 1 μmole glutamine 3 μmole DPN 20 μmoles Na_2SO_4 and 1×10^6 c p m of glucose U C^{14}

or homogenates, which could incorporate S^{35} labeled sulfate or C^{14} gluc into mucopolysaccharide on incubation Since the level of incorporati was small, the identity of the radioactive mucopolysaccharides produc had to be rigorously established This was done by paper chromatograp and paper electrophoresis, with coincidence of the C^{14} and S^{35} radioacti spots of synthesized mucopolysaccharide with those of known chondroit sulfate, and with response to the specific toluidine blue color spray, hy drolysis of the C^{14} mucopolysaccharides and isolation and identification resulting C^{14} hexosamines by ion exchange and paper chromatography ar crystallization of derivatives with carrier glucosamine (Table III), pr cipitation of the C^{14} mucopolysaccharide along with carrier chondroit sulfate by cetylpyridinium bromide, a specific precipitation reagent, an nondialyzability of the precipitated C^{14} mucopolysaccharide, hydrolys of the S^{35} mucopolysaccharide to give labeled sulfate under conditions th lead to hydrolysis of the sulfate group of chondroitin sulfate

Colon segments of deficient and normal rats were incubated with S^{35} sulfate in Krebs Ringer phosphate buffer, pH 7 4 The mucopolysaccharides synthesized and secreted into the medium were isolated by precipitation and purified by paper chromatography, and their radioactivity was determined Incorporation of S^{35} into the mucopolysaccharides by deficient colon segments was found to be about one half compared to that of normal colons When a suspension containing 10 µg of vitamin A was added to the incubation medium of the deficient colons, incorporation was raised to the level of the normal (Table IV) Vitamin A aldehyde was also effective

The conditions under which incorporation of S^{35} sulfate into mucopolysaccharides of rat colon homogenates would take place were first determined Incorporation was highest when the medium contained diphos

TABLE IV

EFFECT OF ADDED VITAMIN A ON INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDES BY COLON SEGMENTS

Vitamin A status	Vitamin A added	Mucopolysaccharide (c p m)
–	—	1279 (4)
–	10 µg (alcohol)	3515 (4)
–	10 µg (aldehyde)	3001 (4)
+	—	2685 (4)

The values are averages of the number of incubations shown in parentheses Incubation period 3½ hours activity added 19 × 10 c p m + = normal rat – = deficient rat

phopyridine nucleotide glucose, glutamine and adenosine triphosphate ("complete system") No incorporation was obtained if the two last named constituents were omitted Homogenates were then prepared from vitamin A deficient and normal rat colons and incubated with the complete system The results are summarized in Table V The S^{35} sulfate incorporated into *mucopolysaccharides by homogenates from deficient colons was once again* about one half that of normal colons The addition of vitamin A suspension to the deficient homogenates restored incorporation to normal The suspending medium alone (serum albumin ethanol) had no effect In Table VI can be seen the specificity tests with other fat soluble vitamins Vitamin A only is capable of completely reversing decreased activity

The next step in this investigation was to localize the function of vitamin A in a specific step in mucopolysaccharide biosynthesis It was found that the vitamin is not involved in the formation of hexosamines because glucosamine or galactosamine, when substituted for glucose and glutamine in the incubation medium of deficient homogenates, could not restore the decrease in incorporation

Next, experiments were undertaken to determine whether vitamin A is required in a step before or after the involvement of the uridine nucleotides in mucopolysaccharide synthesis (see Fig 1) First, a system requiring the uridine nucleotides, and capable of incorporating S^{35} *sulfate into* mucopolysaccharide by rat colon homogenates, was developed This system

TABLE V

INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

Vitamin A status	Vitamin A added (10 μg)	Mucopolysaccharide (c p m)
−	−	346 (3)
−	+	805 (2)
+	−	936 (4)
−	Serum albumin ethanol	268 (2)

[a] The values are averages of the number of incubations shown in parentheses The complete system contained 10 μmoles of ATP 6 μmoles of Mg^{++} 1 μmole of glutamine 3 μmoles of DPN and 10 μmoles of glucose—final volume 1 ml Each incubation contained 10 6 × 10^6 c p m of radioactive sulfate and 14 mg of protein + = normal rat − = deficient rat

TABLE VI

INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES SPECIFICITY OF VITAMIN A

Status of animal	Addition (10 μg)	Mucopolysaccharide (c p m)
Normal	—	896 (2)
Deficient	—	483 (2)
Deficient	Vitamin A alcohol	841 (2)
Deficient	Vitamin D	232 (1)
Deficient	Vitamin E	608 (2)
Deficient	Vitamin E + vitamin A	752 (2)
Deficient	Vitamin K_1	435 (2)

[a] The values are averages of the number of incubations shown in parentheses The complete system was that shown in Table V Each incubation contained 9 78 × 10^6 c p m of activ ty and 14 mg of protein

showed an absolute requirement for glutamine, uridine diphosphoacetyl glucosamine, ane uridine diphosphoglucuronic acid, and a partial requirement for uridine diphosphoglucose and acetylglucosamine When incubated with deficient colon homogenates, it once again showed lowered incorporation of S^{35} sulfate into mucopolysaccharide, restorable with added vitamin A (Table VII) Therefore, vitamin A functions at some step beyond the synthesis of the uridine nucleotides

In an attempt to achieve independence from the use of vitamin A deficient animals, and to find a means of destroying vitamin A *in vitro*, the

colon homogenates were preincubated with lipoxidase, an enzyme known to destroy vitamin A This procedure, as shown in Table VIII, lowered or abolished S^{35} sulfate incorporation Addition of vitamin A restored incorporation only at low concentrations of lipoxidase, presumably because the

TABLE VII

EFFECT OF VITAMIN A ON THE INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES CONTAINING UDP DERIVATIVES

Vitamin A status	Addition of vitamin A 10μg	Mucopolysaccharide (c p m)
+	−	832 (2)
−	−	270
−	+	681

The values are averages of the number of incubations shown in parentheses The complete system consisted of 1 μmole of UDPGA 1 μmole of UDPAG 1 μmole of UDPG 1 μmole of glutamine 8 μmoles of AG (all obtained from Sigma Chemical Corporation) 1 μmole of ATP and 10 μmoles of Mg^{++} Each incubation contained 8 5 × 10^6 c p m of activity and 14 mg of protein + = normal rat − = deficient rat

TABLE VIII

EFFECT OF LIPOXIDASE ON $S^{35}O_4^{--}$ INCORPORATION INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

Preincubation for 1 hour with lipoxidase (3 mg /ml)	Addition of vitamin A 10 μg	Mucopolysaccharide (c p m)
0 01 ml	−	305
0 01 ml	+	538
0 04 ml	−	268
0 04 ml	+	0
0 07 ml	−	0
0 07 ml	+	0

Complete system was that shown in Table V Each incubation contained 10 6 × 10^6 c p m of activity and 14 mg of protein Crystalline soybean lipoxidase was obtained from Nutritional Biochemical Corporation

added vitamin was destroyed by the excess lipoxidase present in the incubation medium

The assay used for mucopolysaccharide synthesis in the above experiments was the uptake of labeled sulfate from the medium into the final product of a many step synthesis Hence, the requirement of vitamin A for any particular step can only be derived by inference One can infer from the results described that the vitamin is not required for the following steps the conversion of glucose to hexosamines, the acetylation of hexosamines, the formation of the uridine diphosphoacetylhexosamines,

the oxidation of uridine diphosphoglucose to uridine diphosphoglucuronic acid By a process of elimination, therefore, one could conclude that vitamin A functions either in the polymerization of the uridine nucleotides, or the activation or transfer of sulfate to the polymer

3 Studies with Pig Colon Enzyme Fractions and Vitamin A

Since rat colon mucosa were difficult to obtain free from muscle and in sufficient quantity, a new approach was sought by the use of pig colon mucosa (Wolf *et al* 1960) In homogenates of that tissue, radioactivity was incorporated into mucopolysaccharide from S^{35} labeled sulfate or C^{14} labeled glucose Mucopolysaccharide was identified in a manner similar to

TABLE IX
INCORPORATION OF S^{35} SULFATE INTO MUCOPOLYSACCHARIDE BY SUBCELLULAR FRACTIONS AND pH 5 ENZYMES OF PIG COLON MUCOSA

Subcellular fraction	Mucopolysaccharide (c p m /mg protein)
Supernatant free from mitochondria and nuclei	1293 (4)
Supernatant free from mitochondria nuclei and microsomes	1521 (4)
Supernatant free from mitochondria nuclei, microsomes and pH 5 enzymes	326 (1)
Microsomes	516 (4)
pH 5 Enzymes	1728 (4)
pH 5 Enzymes and microsomes	1770 (3)

[a] The values are averages of the number of incubations shown in parentheses The complete system consisted of 10 μmoles ATP 6 μmoles $MgCl_2$ 3 μmoles DPN 1 μmole glutamine and 10 μmoles glucose The radioactivity added per incubation 9 × 10^6 c p m made to final volume of 1 ml with phosphate buffer pH 7 4

that described for rat colon mucopolysaccharide The synthesizing activity was located in the supernatant solution, after removal of nuclei, mitochondria, and microsomes from the homogenate (Table IX) and could be precipitated at pH 5 2 This "pH 5 enzyme" fraction contained 48 6% of the total vitamin A content of the mucosa (3 7 μg per 100 gm mucosa) It required uridine triphosphate, adenosine triphosphate, glucose, and glutamine for activity (Table X) and showed a pH maximum between 6 2 and 7 2 This enzyme fraction when obtained from vitamin A deficient pigs was less active (Table XI), lipoxidase also lowered its activity, which in both cases could be restored by added vitamin A (Table XII)

To exclude the involvement of vitamin A in sulfate transfer from 3' phosphoadenosine 5' phosphosulfate ("active sulfate") to the polymer, pH 5 enzyme fraction was incubated with active sulfate and model sulfate acceptors such as chondroitin and nitrophenol No differences were found

TABLE X

REQUIREMENTS OF COFACTORS BY pH 5 ENZYMES FOR INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDES

ATP 10 µmoles	MgCl₂ 6 µmoles	DPN 3 µmoles	Glutamine 1 µmole	Glucose 10 µmoles	UTP 4 µmoles	Mucopolysaccharide (c p m /mg protein)
+	+	+	+	+	−	2234 (2)
−	−	+	+	+	−	2349 (2)
+	+	−	+	+	−	1713 (2)
+	+	+	−	+	−	798 (2)
+	+	+	+	−	−	560 (2)
−	−	+	+	+	+	5723 (2)

The values are averages of the number of incubations shown in the parentheses Radioactivity added per incubation 9×10^4 c p m made to final volume of 1 ml with phosphate buffer pH 7 4

TABLE XI

EFFECT OF VITAMIN A DEFIENCY ON THE INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDE AND SPECIFICITY OF VITAMIN A IN pH 5 ENZYMES

Vitamin A status	*Addition of vitamin A 10 µg*	Mucopolysaccharide (c p m /mg protein)
+	−	2349 (2)
−	−	1407 (2)
−	Vitamin A	2556 (2)
−	Vitamin E	1564 (2)
−	Vitamin D	1530 (2)

The values are averages of the number of incubations shown in the parentheses The complete system consisted of 10 µmoles glucose 1 µmole glutamine 3 µmoles DPN and $S^{35}O_4$ 12×10^6 c p m made up to 1 ml with phosphate buffer pH 6 8

TABLE XII

EFFECT OF PREINCUBATION WITH LIPOXIDASE ON THE INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDE SYNTHESIZED BY pH 5 ENZYMES

Lipoxidase 0 3 mg /ml	Added vitamin A 10 µg	Mucopolysaccharide (c p m /mg protein)
−	−	2706 (4)
0 1 ml	−	1213 (4)
0 1 ml	+	2188 (4)

The values are averages of the number of incubations shown in the parentheses The complete system consisted of 10 µmoles glucose 1 µmole glutamine 3 µmoles DPN and $S^{35}O_4^{--}$ 12×10^6 c p m made up to 1 ml with phosphate buffer pH 6 8

the oxidation of uridine diphosphoglucose to uridine diphosphoglucuronic acid. By a process of elimination, therefore, one could conclude that vitamin A functions either in the polymerization of the uridine nucleotides, or the activation or transfer of sulfate to the polymer.

3. Studies with Pig Colon Enzyme Fractions and Vitamin A

Since rat colon mucosa were difficult to obtain free from muscle and in sufficient quantity, a new approach was sought by the use of *pig colon* mucosa (Wolf *et al.* 1960). In homogenates of that tissue, radioactivity was incorporated into mucopolysaccharide from S^{35}-labeled sulfate or C^{14}-labeled glucose. Mucopolysaccharide was identified in a manner similar to

TABLE IX

INCORPORATION OF S^{35} SULFATE INTO MUCOPOLYSACCHARIDE BY SUBCELLULAR FRACTIONS AND pH 5 ENZYMES OF PIG COLON MUCOSA[a]

Subcellular fraction	Mucopolysaccharide (c.p.m./mg. protein)
Supernatant free from mitochondria and nuclei	1293 (4)
Supernatant free from mitochondria, nuclei, and microsomes	1521 (4)
Supernatant free from mitochondria, nuclei, microsomes and pH 5 enzymes	326 (1)
Microsomes	516 (4)
pH 5 Enzymes	1728 (4)
pH 5 Enzymes and microsomes	1770 (3)

[a] The values are averages of the number of incubations shown in parentheses. The complete system consisted of 10 µmoles ATP, 6 µmoles $MgCl_2$, 3 µmoles DPN, 1 µmole glutamine, and 10 µmoles glucose. The radioactivity added per incubation: 9×10^6 c.p.m., made to final volume of 1 ml. with phosphate buffer, pH 7.4.

that described for rat colon mucopolysaccharide. The synthesizing activity was located in the supernatant solution, after removal of nuclei, mitochondria, and microsomes from the homogenate (Table IX) and could be precipitated at pH 5.2. This "pH 5 enzyme" fraction contained 48.6% of the total vitamin A content of the mucosa (3.7 µg. per 100 gm. mucosa). It required uridine triphosphate, adenosine triphosphate, glucose, and glutamine for activity (Table X) and showed a pH maximum between 6.2 and 7.2. This enzyme fraction when obtained from vitamin A deficient pigs was less active (Table XI); lipoxidase also lowered its activity, which in both cases could be restored by added vitamin A (Table XII).

To exclude the involvement of vitamin A in sulfate transfer from 3'-phosphoadenosine-5'-phosphosulfate ("active sulfate") to the polymer, pH 5 enzyme fraction was incubated with active sulfate and model sulfate acceptors such as chondroitin and nitrophenol. No differences were found

TABLE X

REQUIREMENTS OF COFACTORS BY pH 5 ENZYMES FOR INCORPORATION OF $S^{35}O_4^{-}$ INTO MUCOPOLYSACCHARIDES

ATP 10 μmoles	$MgCl_2$ 6 μmoles	DPN 3 μmoles	Glutamine 1 μmole	Glucose 10 μmoles	UTP 4 μmoles	Mucopolysaccharide (c p m /mg protein)
+	+	+	+	+	−	2234 (2)
−	−	+	+	+	−	2349 (2)
+	+	−	+	+	−	1713 (2)
+	+	+	−	+	−	798 (2)
+	+	+	+	−	−	560 (2)
−	−	+	+	+	+	5723 (2)

The valu s a e a erages of the number of ncubations shown ie the parentheses Radioactivity added per incubat on 9 × 10 c p m made to f nal volume of 1 ml with phosphate buffer pH 7 4

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EFFECT OF VITAMIN A DEFIENCY ON THE INCORPORATION OF $S^{35}O_4^{--}$ INTO MUCOPOLYSACCHARIDE AND SPECIFICITY OF VITAMIN A IN pH 5 ENZYMES

Vitamin A status	Addition of vitamin A 10 μg	Mucopolysaccharide (c p m /mg protein)
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−	Vitamin D	1530 (2)

* The valu s a e a erag s of the n imber of ncubat ons shown in the pa entheses The complete system cons sted of 10 μmol glucos 1 μmole glut mine 3 μmole DPN and $S^{35}O$ 12 × 10 c p m made up to 1 ml w th phosph te buffer pH 6 8

TABLE XII

EFFECT OF PREINCUBATION WITH LIPOXIDASE ON THE INCORPORATION OF $S^{35}O_4^{-}$ INTO MUCOPOLYSACCHARIDE SYNTHESIZED BY pH 5 ENZYMES

Lipoxidase 0 3 mg /ml	Added vitamin A 10 μg	Mucopolysaccharide (c p m /mg protein)
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0 1 ml	−	1213 (4)
0 1 ml	+	2188 (4)

Th val e a e av rag s of the number of incub t ons shown in th e p renth e Th co npl te system cons st d of 10 μmoles glucose 1 μmole glutam ne 3 μmol s DPN and S O 12 × 10 c p m ma le up to 1 ml w th phosphate buff pH 6 8

in sulfate transferase activity between normal and lipoxidase treated fractions, thus excluding vitamin A function in sulfate transfer Such a conclusion, however, has to be treated with caution, since none of the natural acceptors occurring in colon have so far been tried as acceptors in this reaction

On the other hand, evidence has now been obtained (Varandani *et al* 1960) which shows that colon homogenates of deficient rats form phosphoadenosine phosphosulfate less readily than normal homogenates (Table XIII), a lesion which could be corrected by addition of vitamin A One could therefore conclude that vitamin A is involved in mucopolysaccharide biosynthesis at the sulfate activation step

TABLE XIII

EFFECT OF VITAMIN A DEFICIENCY ON $PAPS^{35}$ SYNTHESIS IN RAT COLON HOMOGENATE[a]

Vitamin A status of rats	Addition	$PAPS^{35}$ Expt I	$PAPS^{35}$ Expt II
		(c p m /mg protein)	
Adequate	—	19 200	59 000
Deficient	—	8 440	28,700
Deficient	Vitamin A 20 μg in propylene glycol, 5 μl	24 300	54,100
Deficient	propylene glycol 5 μl	—	31,400

NOTE Activity added Expt I 8 4 × 10⁵ c p m Expt II 16 8 × 10⁵ c p m

[a] Abbreviation $PAPS^{35}$ 3 phosphoadenosine 5 phosphosulfate labeled with S^{35}

4 Vitamin A and Net Synthesis of Mucopolysaccharides in Vitro

Since up to this point only minute amounts of mucopolysaccharide, detectable only by radioactivity, have been synthesized, it was thought necessary to investigate net synthesis of colon mucopolysaccharide *in vitro*, and the effect of vitamin A thereon

Total hexosamine content of rat colon was determined by Moretti and Wolf (unpublished observations), by acid hydrolysis of colon homogenates and isolation and assay of hexosamines Normal colons contained an average of 11 58 ± 1 2 μmoles colons from vitamin A deficient rats an average of 7 38 ± 1 0 μmoles per 100 mg of protein

Actually, "total hexosamine" may include not only that in mucopolysaccharide, but also in the various hexosamine containing intermediates (cf Fig 1) Therefore, it was necessary to separate mucopolysaccharide from low molecular weight material by precipitation and dialysis Again, the deficient colons showed lowered mucopolysaccharide hexosamine to

the extent of about 60% (average of 5 00 ± 0 4 μmoles hexosamines per 100 mg of protein) compared to the normal colon (average of 8 04 ± 0 9 μmoles per 100 mg of protein)

The ratio glucosamine/galactosamine of 0 5 in samples of normal colon was increased to 0 85 in those from deficient animals, hence vitamin A deficiency caused a decrease mainly in the content of galactosamine containing mucopolysaccharides in colon

To study net synthesis of mucopolysaccharide, and the effect of vitamin A thereon a system was developed using rat colon homogenates incubated with glutamine glucose 6 phosphate diphosphopyridine nucleotide, uridine triphosphate, and adenosine triphosphate The samples were assayed for total hexosamine by acid hydrolysis before and after incubation The increase of total hexosamine content after incubation of deficient colon homogenates was about one half compared to that of normal colons The synthetic capacity of the deficient colon homogenates could be restored almost to normal by addition to the incubation mixture of vitamin A The minimum quantity which showed this stimulation was 1 25 × 10^{-2} μmoles (11 5 I U) added per incubation (per 11–13 mg of protein) This action appeared to be not graded but all or none Net synthesis was not restored to normal in deficient homogenates by addition to the incubation mixture of vitamin A acid vitamins E, K_1, and D_2

Again it was necessary to confirm that, since the total hexosamine assayed included the intermediates in mucopolysaccharide biosynthesis, the effect of the vitamin was truly on mucopolysaccharide bound hexosamine

Therefore it was first shown that vitamin A had no effect on the formation of glucosamine 6 phosphate Secondly, mucopolysaccharide was separated from low molecular weight material by precipitation and dialysis

The net formation of mucopolysaccharide bound hexosamine (precipitable and nondialyzable) of normal colon homogenates was between 7 and 12 times greater than that of deficient homogenates The net synthesis of the latter was raised about four fold by addition of vitamin A to the incubation

These results, therefore, definitely establish an effect of vitamin A on the content and net synthesis of mucopolysaccharide in colon

5 Vitamin A and Keratinization

Though not strictly coming under the heading of mucopolysaccharide biosynthesis the theories of vitamin A function in the inhibition of keratinization should here be mentioned Balakhovskii and Drozdova (1957) consider keratinization in vitamin A deficiency to be due to a disturbance of oxidative processes In particular, the catalytic activity of copper in the oxidation of cysteine to cystine which the authors regard as the rate

limiting step in keratinization, was found to be inhibited by carotenoid compounds in model systems The same authors (1956) also found the catalytic action of copper in ascorbinase to be lowered in presence of carotenoids Vitamin A deficient rats excreted more free labeled sulfate than normal rats after injection of S^{35}-methionine (Balakhowskii and Drozdova, 1958), which the authors again assume to be due to a release from the inhibitory action of vitamin A on the copper catalyzed oxidation of SH groups Another interpretation of this result would be to consider it due to increased excretion of sulfate caused by lowered mucopolysaccharide biosynthesis

V Conclusions

From the data presented, especially the tissue culture studies of H B Fell, the conclusion is inescapable that vitamin A has a controlling action on the basal cells of epithelium The presence of vitamin A stimulates them to produce mucus, and its absence permits them to form keratin The vitamin therefore has an action similar to that of a hormone and, in that sense, we know as little about the mode of its biochemical function as about that of other hormones

On the other hand, taking its stimulation of mucus formation as a starting point, results have been presented which show that vitamin A has a direct, possibly coenzymatic, function in the polymerization reaction of the uridine nucleotides to form mucopolysaccharide, or in the sulfation of the latter Perhaps the vitamin could control differentiation of basal cells by being the essential factor in a rate limiting step of a reaction sequence leading to mucus formation

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The Function of Vitamin A in Carbohydrate Metabolism, Its Role in Adrenocorticoid Production

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I Introduction

Although vitamin A was one of the earliest vitamins to be discovered, its function in metabolism in the animal body has not yet been elucidated Wald (1953) and his group have described in a brilliant series of papers, the mechanism whereby vitamin A functions in vision However, this can not be the only function of vitamin A since animals die from vitamin A deficiency but not from blindness The field has been reviewed by Lowe and Morton (1956), and only those papers pertaining to carbohydrate metabolism and the adrenoglucocorticoids will be referred to here

In early work seeking a metabolic function of the vitamin, von Euler and Schmidt (1934) reported an increase in purine content in growing tissues after vitamin A administration to vitamin A depleted animals and on this basis concluded that the principal role of vitamin A was the stimulation of the building of cells through a possible role in nucleic acid metabolism

Von Euler has also suggested a possible connection of vitamin A with oxidation reactions (von Euler and Ahlstrom, 1932), finding an increased oxygen consumption in liver tissue with increased amounts of vitamin A present Sure and DeWitt (1938) have also reported a somewhat lowered oxygen uptake *in vitro* in vitamin A deficiency On the other hand, Sadhu and Brody (1947) reported that the oxygen consumption of liver slices from rats with hypervitaminosis is again lower than that of normal controls and that, in general, hypometabolism is produced by hypervitaminosis A

Also Blaizot and Serfaty (1955) have shown that the O_2 uptake of diaphragms from vitamin A deficient rats is greater than that of control animals, and this is in agreement with the report of Ray and Sadhu (1959) that rat diaphragm from animals fed excess levels of vitamin A showed a decreased utilization of glucose and a decrease in glycogen synthesis These authors attribute these findings to a widespread depression in metabolism induced by hypervitaminosis A

In his review (1942), Rosenberg states that vitamin A seems to exert some nonspecific function upon fat and carbohydrate metabolism, stating that the fat, cholesterol, and glycogen content of the organism decreases during vitamin A deficiency, whereas excess doses cause an increase It seems probable that at least some of these changes are due to inanition, however, Brown and Morgan (1948) have found total carcass fat to be decreased in vitamin A deficient rats as compared to pair fed rats Green *et al* (1955) found no effect of vitamin A deficiency on cholesterol levels, and Ray and Sadhu (1959) have reported that hypervitaminosis A caused a decrease in liver glycogen and an increase in liver fat

Rather than study the change in level of a particular metabolite, we decided to use a more dynamic approach to the problem of vitamin A function by first searching for a metabolic block in the intact animal In this search for possible leads as to where vitamin A might function in the general metabolic scheme, radioacetate was used as a general metabolic precursor and was administered to A deficient and pair fed normal rats Pair feeding was used throughout the intact animal work to avoid inanition effects This work led us directly to a role of vitamin A in carbohydrate metabolism as indicated by a requirement of vitamin A for glycogen synthesis This proved to be an indirect effect mediated by the glucogenic hormones of the adrenal gland, and it is principally this work which will be presented in this paper

In many experiments to be reported work was first done with very severely vitamin A deficient animals, and at times the results obtained were later compared with those obtainable with more mildly deficient animals to rule out the changes due to general debility and morbidity Only those

changes observable also in the mildly deficient animal are thought to be indicative of a real metabolic function of vitamin A

II Vitamin A and Glycogen Synthesis

1 Vitamin A Deficiency and Acetate Metabolism

a Acetate Conversion to CO It was found that vitamin A deficiency was without effect on the rate of acetate oxidation to CO_2 indicating normal functioning of the tricarboxylic acid cycle in the vitamin A deficient animal

b Acetate Conversion to Amino Acids and Protein Similarly the incorporation of radioactivity into liver protein in 4 hour experiments indicated no difference between the pair fed A deficient animal and the normal with regard to the activity of the liver protein, liver alanine, or liver aspartic acid Since the major labeling in liver protein is due to the amino acids derived from the tricarboxylic acid cycle especially glutamic and aspartic acids this also indicates a lack of effect of vitamin A on the tricarboxylic acid cycle This would also indicate that there was no effect of vitamin A deficiency on the connecting link between the tricarboxylic acid cycle and glycolysis, since there was no effect of vitamin A deficiency on the incorporation of acetate C^{14} into alanine and aspartic acid (representative of pyruvate and oxalacetate, respectively)

c Acetate Conversion to Cholesterol In the case of cholesterol a greater total incorporation of acetate C^{14} was found in the vitamin A deficient rat than in the pair fed control This may be due to a block in some other pathway of acetate metabolism caused by vitamin A deficiency and thus leading to an accumulation of intermediates used in cholesterol biosynthesis At any rate, it is apparent that vitamin A is not required for cholesterol biosynthesis and this agrees with the finding of Green and Morton (1955) that liver and plasma cholesterol levels are not affected by vitamin A deficiency

d Acetate Conversion to Fatty Acids Again, in the case of fatty acids as in cholesterol, the incorporation was somewhat greater in the deficient animals indicating again that vitamin A does not function in fatty acid synthesis

e Acetate Conversion to Glycerol In this case also no effect of vitamin A deficiency was found indicating no effect of vitamin A on the pathway from acetate to triose

f Acetate Conversion to Glycogen Data on the incorporation of acetate C^{14} into liver glycogen are given in Table I In this experiment each animal was given 600 mg of nonradioactive glucose 30 minutes before administration of the radioacetate and the animals were killed 4 hours after the

acetate injection As can be seen, the specific activity of the glycogen isolated from the pair fed control animals was approximately eight times greater and the total radioactivity about ten times greater than that of the glycogen isolated from the deficient animals These results indicated a pronounced effect of vitamin A deficiency on the reversal of glycolysis (Lane *et al*, 1956)

TABLE I

ACETATE *1* C^{14} INCORPORATION INTO LIVER GLYCOGEN

	Vitamin A deficient rat	Pair fed rat
Glycogen		
Specific activity, $\mu c \times 10^{-3}$ per mg	0 59	4 45
Total quantity isolated mg	74	104
Total C^{14} activity incorporated, μc	0 044	0 464

TABLE II

GLUCOSE *1* C^{14} INCORPORATION INTO LIVER GLYCOGEN AND ALANINE

	Vitamin A deficient rat	Pair fed rat
Glycogen		
Specific activity $\mu c \times 10^{-3}$ per mg	24 5	8 5
Total quantity isolated, mg	87	289
Total activity incorporated, μc	2 14	2 40
Alanine		
Specific activity $\mu c \times 10^{-3}$ per mg	0 739	0 248
Total μc per gm protein	0 035	0 012

2 Glycogen Formation from Other Precursors In Vivo *Studies*

a Glycogen from Glucose A block in glycogen synthesis was observed in the first vitamin A deficient animals which had been examined, no glycogen being found in their livers However, after the usual glucose injections, glycogen was formed in an amount similar to that formed in the pair fed controls (Wolf *et al*, 1957b) In confirmation of this, when glucose C^{14} was used as glycogen precursor, no effect of the deficiency on incorporation into liver glycogen was found, the total activity incorporated being essentially the same in both groups of animals, as shown in Table II

b Glycogen from Lactate In order to look directly at the reversal of glycolysis, experiments were carried out with labeled lactate and labeled glycerol From the data in Table III it can be seen that the total activity incorporated from lactate is reduced to approximately one fifth in the vitamin A deficient animal

c Glycogen from Glycerol The data in Table III also indicate that incorporation of glycerol C^{14}, serving as a stand in for triose, into liver glycogen was markedly depressed by vitamin A deficiency

From these experiments it can be concluded that in the intact animal vitamin A deficiency interferes with glyconeogenesis at a step between triose and glucose However, this interference is in only one direction, that of glucose synthesis, since the incorporation of radioactivity from glucose into alanine (Table II) was not affected

3 Glycogen Formation from Other Precursors In Vitro *Studies*

a In Rat Liver Slices From Glucose and Fructose Since the effects reported in the intact animal might be of an indirect nature, for example, caused by an effect on cell permeability or on the formation of a glycolysis

TABLE III

INCORPORATION INTO LIVER GLYCOGEN

	Vitamin A deficient rat	Pair fed rat
From lactate 1 C^{14}		
Specific activity $\mu c \times 10^{-3}$ per mg	0.43	1.93
Total quantity isolated mg	140	163
Total activity incorporated μc	0.060	0.315
From glycerol *1 3* C^{14}		
Specific activity $\mu c \times 10^{-3}$ per mg	0.378	7.3[illegible]
Total quantity isolated mg	100	86.2
Total activity incorporated μc	0.038	0.63

controlling hormone experiments were carried out *in vitro*, using rat liver slices and rat liver homogenates Table IV gives the data for the rat liver slice experiments

From this table it can be seen that the conversion of both glucose and fructose to glycogen was markedly affected in these animals by vitamin A deficiency This was surprising, since *in vivo* the deficiency had been found to have no effect on glycogen synthesis from glucose, therefore two further experiments were carried out using in one case, mildly deficient animals which had, however, already shown body weight loss due to vitamin A deficiency and in the other case severely deficient animals as had been used in the liver slice experiment From the results given in Table V it can be seen that, although glycogen biosynthesis was markedly depressed in the severely deficient animals there was little effect in the mildly deficient animals

The data in Table IV indicate that in rat liver slices, fructose (taken as representative of triose phosphate on the basis of the work of Hers and

Kusaka (1953) and Leuthardt *et al* (1953) that fructose in liver is converted to fructose 1-phosphate and then to dihydroxyacetone phosphate and glycerylaldehyde) conversion to glycogen is not affected by vitamin A deficiency This is in direct contrast to the work with intact animals, which did indicate an effect of vitamin A deficiency in the metabolic pathway between triose and glucose

TABLE IV

GLYCOGEN FORMATION BY RAT LIVER SLICES

Conditions	Weight of liver glycogen (mg)	*Specific activity of liver glycogen with added carrier* (dis/minute/mg)	*Calculated* specific activity of undiluted liver glycogen (dis/minute/mg)	Total activity of liver glycogen (dis/minute)
From glucose C^{14}				
Deficient in vitamin A	*6 79*	*526*	*2979*	*20 210*
Pair fed normal	17 43	849	2323	40 500
Deficient in vitamin A	2 85	314	3255	9 420
Pair fed normal	10 39	632	2615	27,160
From fructose C^{14}				
Deficient in vitamin A	6 00	490	2960	17,630
Pair fed normal	*17 00*	*1040*	*2878*	*48,900*

[a] Incubation of 400-700-mg slices in 4 ml. KCl $CaCl_2$-$MgCl_2$-$KHCO_3$ buffer at pH 7 4 Additions [illegible] μc glucose *1* C^{14} (80 μM) or 6 5 μc fructose *U* C^{14}

TABLE V

INCORPORATION OF GLUCOSE *1* C^{14} INTO GLYCOGEN BY RAT LIVER SLICES

Vitamin A status	Glycogen specific activity (d p m /mg)
Mildly deficient	667
Mildly deficient	620
Mildly deficient	569
Vitamin A normal	*746*
Vitamin A normal	926
Vitamin A normal	1372
Severely deficient	76
Vitamin A normal	811

[a] The livers were removed and sliced with a Stadie Riggs tissue slicer The tissue was incubated in Krebs Ringer phosphate buffer with *0 3 μc glucose 1* C^{14}

These *in vivo* data (supported by that below on the liver homogenate metabolism of the fructose phosphates) demonstrate (1) that the *in vivo* block between triose and glucose 6 phosphate does not exist *in vitro* (nor between fructose 1,6 diphosphate and glucose) and is probably, therefore hormone controlled as demonstrated later, and (2) that in the severely vitamin A deficient rat the *in vitro* conversion of glucose 6 phosphate (whether derived from glucose or fructose) to glycogen is depressed, presumably owing to nonspecific debilitation in the late stages of the deficiency

TABLE VI

GLUCOSE FORMATION FROM FRUCTOSE AND FRUCTOSE PHOSPHATE IN LIVER HOMOGENATES

Substrate used	Amount used (μmoles)	Glucose formed					
		Normal liver			Deficient liver		
		Amount (μmoles)	Radioactivity (c p m × 10^3)	Yield (%)	Amount (μmoles)	Radioactivity (c p m × 10^3)	Yield (%)
Fructose	27	9 4	—	33 8	8 5	—	30 6
Fructose C^{14}	27 (6 5 × 10^5 c p m)	5 8	193	29 7	5 7	196	30 2
Fructose 1 6 diphosphate	10	8 3	—	83 4	8 3	—	83 4
Fructose 6 phosphate	10	4 4	—	44 5	3 9	—	38 9

b In Rat Liver Homogenates from Fructose Experiments on the conversion of C^{14} fructose and the fructose phosphates to C^{14} glucose were then carried out with liver homogenates The data, presented in Table VI, show no significant differences in glucose formation between normal and vitamin A deficient liver homogenates The lack of effect on the conversion from fructose 1 6 diphosphate indicates that there is no inactivation of liver fructose 1,6 diphosphatase in vitamin A deficiency Mokrasch *et al* (1956) have reported that cortisone administration increases the amount of liver fructose 1 6 diphosphatase It will be pointed out below that vitamin A causes a deficiency in glucocorticoid hormone production in the intact animal, but these data would indicate that a deficiency in this hormone does not decrease the amount of the enzymes involved in the pathway of glucose synthesis

to incorporate radioactivity from acetate into glycogen However, when given cortisone even in the absence of vitamin A, glycogen was produced normally As would be expected, in the negative controls without vitamin A and cortisone, no glycogen was formed, and with both, glycogen formation was unaffected

In an experiment of long duration, weanling rats were adrenalectomized and put on either cortisone treatment with vitamin A deficiency or vitamin A treatment and no cortisone administration and fed for 32 days As can be seen from the data in Table IX, in the absence of adrenal glands and without cortisone, but with the administration of vitamin A, the rats grew normally and showed no symptoms of the deficiency except in the depression of glycogen labeling, whereas the animals receiving cortisone but no vitamin A came down with all the symptoms of vitamin A deficiency, yet produced glucose normally These data would indicate that vitamin A deficiency leads to a change in the adrenal gland such that it is unable to produce glucogenic hormone

TABLE IX

GLYCOGEN FORMATION AND ADRENALECTOMY IN WEANLING RATS

Adrenals[b]	Cortisone treatment	Vitamin A treatment	Initial weight (gm)	Final weight (gm)	Weight gain (gm)	Weight of liver glycogen (mg)	Specific activity of liver glycogen (c p m / mg)	Total activity of liver glycogen (c p m)
−	−	+	36	120	+84	187 0	96 4	18,020
−	+	−	38	94	+56	62 5	2040	127 500
+	−	+	40	122	+82	203 0	669	135 900

The rats were adrenalectomized where indicated at weaning They were given vitamin A in the diet in amounts as described (Wolf *et al* 1957) or alternatively given cortisone intraperitoneally (5 mg /day) After 32 days they received 10 0 × 10⁶ c p m of acetate *1 C*¹⁴ 0 minutes after receiving 300 mg of nonradioactive glucose

[b] + = Present − = absent

c *Adrenocorticotropic Hormone (ACTH) Treatment* In order to eliminate the possibility that vitamin A and cortisone might influence glyconeogenesis independently, an experiment with ACTH, instead of cortisone, treatment was carried out If our postulation is correct, that vitamin A deficiency does produce a "chemical adrenalectomy" as far as glucocorticoid production is concerned (Wolf *et al* , 1958a) then ACTH, which normally causes an increased secretion of these hormones, should be unable to do so in the A deficient animal and would then have no effect on the restoration of

glyconeogenesis If, on the other hand, vitamin A deficiency depresses glyconeogenesis by one mechanism, and cortisone treatment increases it by an independent one, then ACTH should increase glyconeogenesis even in the presence of a vitamin A deficiency The results shown in Table X clearly demonstrate that vitamin A deficiency destroyed the ability of the adrenal cortex to produce glucocorticoid hormone Incorporation of acetate C^{14} into liver glycogen was lowered by vitamin A deficiency (rat 2) as much as by adrenalectomy (rat 1), in spite of simultaneous ACTH treatment in both cases

TABLE X
GLYCOGEN FORMATION AND ACTH TREATMENT[a]

Rat No	Adrenals[b]	ACTH treatment	Vitamin A status	Initial weight (gm)	Final weight (gm)	Weight difference (gm)	Weight of liver glycogen (mg)	Specific activity of liver glycogen (c p m / mg)	Total activity of liver glycogen (c p m)
1	−	+	+	182	205	+23	134 6	16 3	2 200
2	+	+	−	111	76	−35	36 9	24 9	920
3	+	+	Slightly deficient	156	145	−11	88 7	52 4	4,640
4	+	+	+	190	207	+17	249 5	60 6	15 120
5	+	+	+	193	217	+24	147 0	52 8	7 760
6	+	−	+	159	179	+20	155 6	60 2	9 360

[a] Vitamin A deficient and normal rats were treated with ACTH for 9 days after adrenalectomy where indicated then given 300 mg of nonradioactive glucose and after 30 minutes 8 0 × 10 c p m of acetate 1 C^{14}
[b] + = Present − = absent

d Time of Production of Deficiency A time study was undertaken in which weanling rats fed an A deficient diet were compared with pair fed controls fed a complete ration and killed at different stages of the deficiency In each case glucose and acetate C^{14} were given prior to killing to study incorporation into glycogen As shown in Table XI the loss in ability to synthesize glucose appears very early, at the time when the growth curve has just begun to flatten and almost complete loss of glyconeogenesis occurred only 2 days after the first weight loss had been observed

e Adrenal Histology Lowe et al (1953) have observed a depression in phospholipid content of the zona fasciculata of the adrenal cortex in vitamin A deficient animals For that reason the adrenals of the animals described in Table XI were examined histologically it was found that the

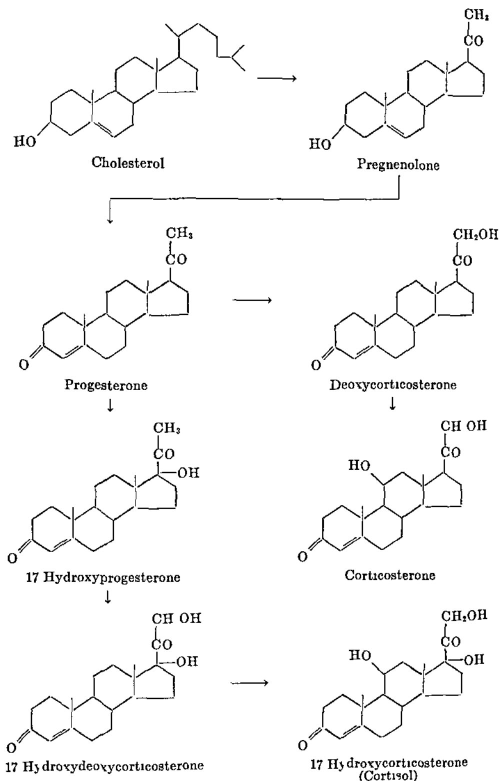


FIG 1 Pathway of glucocorticoid biosynthesis

progesterone being (1) 17 hydroxylation followed by 21 hydroxylation of progesterone to 11 deoxycortisol followed by 11 β hydroxylation to cortisol, and (2) 21 hydroxylation of progesterone to 11 deoxycorticosterone followed by 11 β hydroxylation to corticosterone These reactions are brought about by the series of hydroxylating enzymes (a) 17α hydroxylase, (b) 21 hydroxylase, and (c) 11β hydroxylase all of which require TPNH and molecular oxygen [the work of Tomkins *et al* (1957, 1958) has indicated a requirement for an unidentified cofactor for the 11β hydroxylase step]

In the rat and rabbit, corticosterone is the predominant adrenal vein steroid, but in man, monkey, cat, and dog cortisol predominates (cf Hechter and Pincus, 1954)

The immediate precursor of progesterone is Δ^5 pregnenolone which is produced in adrenal tissue as a result of degradation of the side chain of cholesterol Thus, to determine the site of action of vitamin A in corticosterone biosynthesis it was necessary to examine the steps from mevalonic acid to cholesterol (that from acetate to cholesterol had already proved to be unaffected by vitamin A deficiency) and from cholesterol to progesterone, the synthesis of DOC and the corresponding 17 OH compound, and the step from DOC to corticosterone As before, severely deficient animals were used first, and then the pathways affected in the adrenals from severely deficient animals were later checked with adrenals from mildly deficient animals

2 Corticoid Production in Quartered Adrenals

a Effect of Vitamin A Deficiency In order to explore further the possibility of a direct effect of vitamin A deficiency on glucocorticoid production, *in vitro* experiments were carried out with quartered adrenals from vitamin A deficient and vitamin A normal animals In these experiments, quartered adrenal glands were incubated with ACTH triphosphopyridine nucleotide (TPN), and glucose and the steroids secreted into the medium were measured by the tetrazolium blue reaction and assumed to be principally corticosterone on the basis of the report by Koritz and Péron (1959) that the 21 hydroxy adrenal steroids (the tetrazolium responsive steroids) produced by rat adrenal tissue consist mainly of corticosterone

Table XIII shows that vitamin A deficiency caused corticosterone secretion into the medium to drop to about half of the normal (Van Dyke and Wolf, 1958) This is in direct substantiation of the intact animal work mentioned above (Section II, 5)

Table XIV indicates the reduction in these 21 hydroxy adrenal steroids to be specific for vitamin A, and not brought about by inanition or starvation or by vitamin E deficiency

The effect of vitamin A added directly to the incubation media cou not be studied, since this substance interfered with the tetrazolium assa However, curative experiments with vitamin A given to the animal pri

TABLE XIII
EFFECT OF VITAMIN A DEFICIENCY ON CORTICOID PRODUCTION BY QUARTERED RAT ADRENAL GLANDS

Adrenals of normal rats[b]		Adrenals of deficient rats[b]	
Corticoids produced (μg)	Corticoid/wet weight of glands (μg /100 mg)	Corticoids produced (μg)	Corticoid/wet weight of glands (μg /100 mg)
33	23 4	9 4	6 6
30	21 4	8 7	6 1
23	21 9	13 0	10 3
33	23 9	15 0	11 7

[a] Quartered adrenal glands from three rats were combined preincubated as described in the text and incu bated for 1 hour at 38 C under 95 % O_2 + 5 % CO_2 in 3 ml Krebs Ringer phosphate buffer pH 7 6 with mg glucose 1 mg ACTH and 2 3 μmoles TPN ACTH as Adrenomone Armour—ACTH in gelatin
[b] Each value represents three rats

TABLE XIV
SPECIFICITY OF EFFECT OF VITAMIN A DEFICIENCY ON CORTICOID PRODUCTION BY QUARTERED RAT ADRENAL GLANDS

Adrenals from	Corticoids produced (μg)
Vitamin A deficient rats	16 5
Vitamin E deficient rats	28 4
Normal rats (pair fed)[b]	27 8
Normal rats (fed ad libitum)	31 4
Starved rats	29 5

[a] Quartered adrenal glands from three rats were combined preincubated as described in the text then in cubated for 1 hour at 38 C under 95 % O + 5 % CO_2 in 3 ml Krebs Ringer phosphate buffer pH 7 6 with 6 mg glucose and 1 mg ACTH To each incubation was added 2 3 μmoles TPN
[b] Pair fed with vitamin A deficient rats
Starved for 5 days with water ad libitum

to killing are shown in Table XV and demonstrate that the ability of the gland to produce corticosterone had been restored essentially to normal 4 hours after the vitamin A injection

b Effect of TPNH Table XVI indicates that TPNH or TPN together with glucose 6 phosphate (G 6 P) markedly stimulates corticoid production in the adrenals from vitamin A deficient rats (Van Dyke *et al* , 1959)

In all cases, both in this experiment and in others, while TPNH and TPNH generating systems did have a marked effect on corticoid production

TABLE XV

EFFECT OF PREINJECTION OF VITAMIN A ON CORTICOID PRODUCTION BY QUARTERED RAT ADRENAL GLANDS

Vitamin A status	*Hours after vitamin A injection*	*Total corticoid produced (μg)*
Normal	0	30
Deficient	0	18
Deficient	2	20
Deficient	4	27
Deficient	6	26

Vitamin A (15 mg) suspended in saline was injected at the hours indicated prior to killing of the animals and excision of adrenal glands Quartered adrenal glands from three rats were combined preincubated as described in the text then incubated for 1 hour at 38 C under 95 % O_2 + 5 % CO in 3 ml Krebs Ringer phosphate buffer pH 7 6 with 6 mg glucose and 1 mg ACTH To each incubation was added 2 3 μmoles TPN

TABLE XVI

EFFECT OF TPNH AND GLUCOSE 6 PHOSPHATE ON CORTICOID PRODUCTION (TOTAL WEIGHT) BY QUARTERED ADRENAL GLANDS FROM NORMAL AND VITAMIN A DEFICIENT RATS

Addition	*Adrenals of normal rats*		*Adrenals of deficient rats*	
	Total corticoid produced mean values (μg)	Range (μg)	Total corticoid produced mean values (μg)	Range (μg)
TPN 2 3 μmoles	28 1[b]	25 0–32 0	16 5[b]	13 4–18 0
TPNH 4 6 μmoles	27 2	25 1–30 1	22 0	20 2–24 0
Glucose 6 phosphate 0 8 μmoles and TPN 2 3 μmoles	40 0	37 2–50 4	34 0	30 7–36 1

Quartered adrenal glands from three rats were combined preincubated as described in the text then incubated for 1 hour at 38 C under 95 % O + 5 % CO in 3 ml Krebs Ringer phosphate buffer pH 7 6 with 6 mg glucose and 1 mg ACTH The following were added where indicated TPN 3 μmole TPNH 4 6 μmoles glucose 6 phosphate 0 5 μmoles Mean values and range given are for six experiments each (18 animals per value)

[b] Difference between normal and deficient significant (<0 01%)

by vitamin A deficient adrenals, they did not restore activity to that of the normals, and the question thus arose whether vitamin A was exerting its effect on the animal through an effect on a TPNH production system or whether this observed effect of TPNH in the quartered adrenal was due

perhaps to a "potentiation" of some remaining adrenal cells and not specifically related to vitamin A function

3 Vitamin A Deficiency and TPNH Production

At this point it appeared that the effect of vitamin A deficiency, and hence the action of vitamin A, could be either directly on a reaction in the pathway of corticosterone biosynthesis or that the effect of the deficiency could be on the production of a cofactor required for that biosynthesis The latter possibility was considered first because of the results obtained with TPNH, which indicated that vitamin A might function in one of the enzymes involved in the reduction of TPN in the adrenal A systematic examination was made of the enzymes known to be involved in TPNH production, studying their activities in the vitamin A deficient and normal animal (Van Dyke, 1960) The following enzymes were assayed in adrenal homogenates from pairs of deficient and normal rats (1) *G 6 P dehydrogenase* This enzyme was assayed *in vivo* by comparing the ratio of radioactivity expired in the CO_2 from glucose *1* C^{14} and glucose *6* C^{14} No differences were found in the ratio between deficient and normal rats, and the results indicated that neither glucose 6 phosphate (G 6 P) dehydrogenase nor 6 phosphogluconate dehydrogenase are affected by the deficiency (2) *Hexokinase* No difference could be found in the rates of glucose 6 phosphate production from glucose and ATP This finding leads to the paradox that G 6 P plus TPN stimulated corticosterone production in the quartered adrenal, whereas glucose plus TPN did not, despite the fact that hexokinase levels are normal This may possibly be explained by a penetration barrier to glucose but not to G 6 P (3) A combined assay for *G 6 P dehydrogenase* and *hexokinase* showed no difference between deficient and normal adrenal homogenates in the rate of reduction of TPN with glucose as substrate (4) In a combined assay for *phosphorylase, phosphoglucomutase,* and *G-6 P dehydrogenase,* no differences were found in the rate of reduction of TPN in adrenal homognates when glycogen served as substrate (5) *Phosphorylase,* when assayed independently, was found to be unaffected by the deficiency (6) *Transhydrogenase* Vignais (1957) has reported a depression in liver transhydrogenase activity in vitamin A deficiency, an effect which he has stated is obtainable only *in vivo* (Vignais, 1958) This could possibly be related to the findings of Talalay and Williams Ashman (1958) that steroid hormones activate the transfer of hydrogen between the pyridine nucleotides Thus if vitamin A functions in the adrenal production of steroids, these could affect the activity of liver transhydrogenase

Transhydrogenase activity in the adrenal was tested in two different experiments comparing the activity of TPNH with that of TPN plus DPNH as compared to TPN alone on the production of corticosterone from DOC

in vitamin A deficient pig adrenal mitochondria, and on total corticoid production by quartered adrenals from vitamin A deficient rats. In both cases there was no less steroid produced in the presence of DPNH and TPN than in the presence of TPNH, indicating that the vitamin A deficiency had not interfered with transhydrogenase activity.

4. Glycogen Production from Uridine Diphosphoglucose (UDPG)

Leloir *et al.* (1959) have found that glycogen synthesis in the liver can take place by way of UDP glucose as well as by reversal of glycolysis. Because our work on the role of vitamin A in mucopolysaccharide biosynthesis,

TABLE XVII
GLYCOGEN PRODUCTION IN RAT ADRENAL TISSUE USING UDPG AS AN INTERMEDIATE

Vitamin A status	Cofactors	Glycogen produced (μg)
−	—	2050
−	UDPG	2600
−	UDPG	2537
−	UTP	1886
− (at zero time)	UDPG	1527
+	UDPG	2512
+	UDPG	2862
+	UTP	2662
+ (at zero time)	UDPG	2000
+	—	2175

Buffered at pH 8.4 with Tris maleate buffer. Cofactors added as follows: UDPG, 0.5 μmoles; UTP, 0.5 μmoles; glucose 6-phosphate, 0.5 μmoles. Glucose 6-phosphate was added to each incubation. Reaction stopped with 5% trichloroacetic acid.

reported elsewhere in this Symposium, indicated the possibility of a function at a polymerization step of uridine nucleotides, it appeared possible that there still might be a direct role of vitamin A in the adrenal in glycogen synthesis not mediated through a glucocorticoid, the glycogen so synthesized then functioning in the stimulation of glucocorticoid hormone synthesis. Adrenal homogenates from normal and vitamin A deficient rats were incubated with UDPG. The results of this experiment are given in Table XVII, and as can be seen there was essentially no difference in glycogen production between the vitamin A deficient and the vitamin A normal adrenals.

In an experiment with normal pig adrenals in which vitamin A was destroyed by lipoxidase treatment, again no effect of vitamin A on glycogen production from UDPG was found.

5 *Effect of Vitamin A Deficiency on Corticoid Synthesis from C[14] Cholesterol in Adrenal Homogenates*

a Corticosterone Biosynthesis (1) *In pig adrenal homogenates* Since we had been unable to find an effect of vitamin A on the production of the TPNH cofactor required in corticoid biosynthesis, we returned at this stage to a study of the possible role of vitamin A directly in a reaction in the pathway of corticosterone biosynthesis In most of these experiments designed to determine the step impaired in vitamin A deficiency, cholesterol C^{14} was used as precursor of the adrenal steroids In all experiments the same weight of adrenal homogenate was used for both the deficient and normal adrenal and total net synthesis is reported

TABLE XVIII
LABELED STEROIDS PRODUCED BY PIG ADRENAL TISSUE USING CHOLESTEROL C^{14} AS A PRECURSOR

Steroid	Activity found in deficient tissue (d p m)	Activity found in normal tissue (d p m)
Corticosterone	43 428	88,620
Progesterone	18 312	60 300
DOC	9,908	38,488
17 OH DOC	27 832	—[b]

[a] Tissue was homogenized and incubated in Sorenson's phosphate buffer pH 7 2 with the following additions sucrose 0 05 *M* KCl 0 154 *M* NaCl 0 154 *M* niacinamide 5 m*M* sodium fumarate 5 m*M* $MgSO_4$ 5 m*M* glucose 0 01 *M* Cofactors added ATP 1 0 m*M* DPN 0 5 m*M* TPN 0 5 m*M* Incubated for hours under 95 % O_2 + 5 % CO_2 Values represent an average of three experiments I 95 μc cholesterol-4-C^{14} added

[b] Undetectable

In one experiment in this series vitamin A deficient pigs were used, and the results of this experiment are given in Table XVIII These data show a depression in the formation of the 17 deoxy steroids and an increase in the 17 hydroxy DOC in vitamin A deficiency

This experiment was repeated, using rat adrenal tissue, and again, as can be seen in Table XIX, there was a depression in the activity incorporated into progesterone and DOC, but in this case the principal difference was in the labeling of corticosterone

2 In rat adrenal homogenate In a second experiment with rat adrenals, therefore, corticosterone production alone was considered In this experiment TPNH generating systems were also added As can be seen from the data in Table XX, however, these additions had no effect on corticosterone production in the deficient adrenals, although they did increase the corticosterone production of the normal adrenals by approximately 20 %

When the levels of some other steroids were examined in this rat adrenal experiment it was found that, as in the case of the pig adrenals, there was a marked accumulation of 17 hydroxy DOC in the deficient adrenals, but none could be detected in the normal (Van Dyke *et al*, 1960a) The results of these data are given in Table XXI It is interesting to note from these data, comparing Tables XX and XXI that, although a TPNH generating

TABLE XIX
LABELED STEROIDS PRODUCED BY RAT ADRENAL TISSUE USING CHOLESTEROL C^{14} AS THE PRECURSOR

Steroid	Activity found in severely deficient tissue (d p m)	Activity found in normal tissue (d p m)
Corticosterone	52 672	97 888
Progesterone	19 840	27 144
DOC	13 228	18 096

[a] Conditions of incubation were the same as those in Table XVIII Cofactors added ATP 1 0 mM DPN 0 5 mM TPN 0 5 mM The values represent an average of three experiments

TABLE XX
CORTICOSTERONE PRODUCED UNDER CONDITIONS OF INCREASED TPNH PRODUCTION

Addition	Corticosterone produced in deficient (d p m)	Corticosterone produced in normal (d p m)
Homogenate	2650	7458
Homogenate + glucose 6 phosphate	3104	8552
Homogenate + glucose 6 phosphate + glucose 6 phosphate dehydrogenase	2412	9196

Conditions of incubation were the same as those given in Table XVIII Cofactors added ATP 1 0 mM DPN 0 5 mM TPN 0 5 mM glucose 6 phosphate 0 5 mM Three units of glucose 6 phosphate dehydrogenase were added

system had no effect on corticosterone production in the deficient adrenal, it did have a marked effect on 17 hydroxy DOC production Thus the increase in total steroids found upon TPNH addition in quartered deficient adrenals (Section III, 2, b) could be accounted for as 17 hydroxy DOC

3 Effect of severity of vitamin A deficiency The data in Table XIX indicated an effect of vitamin A deficiency on the production of not only corticosterone, but also progesterone and DOC Since the animals used in this experiment had been severely vitamin A deficient, a further experiment

was carried out with mildly deficient animals in an attempt to find out which step was blocked first The data from this experiment are given in Table XXII and show clearly that in the mildly deficient animal, while corticosterone synthesis is still slightly inhibited, there is as yet no effect of the deficiency on the production of progesterone or DOC

TABLE XXI

17 HYDROXY DOC PRODUCED UNDER CONDITIONS OF INCREASED TPNH PRODUCTION

Additions	17 OH DOC produced in deficient (d p m)	17 OH DOC produced in normal (d p m)
Homogenate	2688	—[b]
Homogenate + glucose 6 phosphate + glucose 6 phosphate dehydrogenase	5376	—[b]

[a] Same experiment as given in Table XX
[b] Undetectable

TABLE XXII

LABELED STEROIDS PRODUCED WHEN RAT ADRENAL TISSUE WAS INCUBATED WITH CHOLESTEROL C^{14}

Enzyme source and additions	Mildly deficient		Normal	
	Corticosterone (d p m)	Progesterone (d p m)	Corticosterone (d p m)	Progesterone (d p m)
1 Whole homogenate	2190	—	3660	—
1 Mitochondria	—	11538	—	9312
2 Mitochondria	—	5906	—	3160
2 Mitochondria + pregnenolone	—	3856	—	2094

[a] Conditions of incubations of whole homogenate were the same as those given in Table XVIII For the incubation of the mitochondria glucose 6 phosphate 0 5 mM and glucose 6 phosphate dehydrogenase 3 units were added in addition to the other cofactors as given in Table XVIII Experiment 1 had 0 65 μc cholesterol 4 C^{14} added and experiment 2 had 0 35 μc cholesterol 4 C^{14}

b Progesterone Biosynthesis The possibility that vitamin A might be concerned with the synthesis of steroid hormones, particularly progestins, was first proposed by Lamming and Salisbury in 1952, on the basis of their finding that progesterone prevented abortion in vitamin A deficient rabbits

Grangaud and Conquy (1958a, b c) have reported a role of vitamin A in progesterone biosynthesis from pregnenolone They found that progesterone given to vitamin A deficient rats caused a slight resumption in weight gain and brought about the re establishment of normal estrus in some rats, although it was without effect on the xerophthalmia of vitamin A deficiency

Pregnenolone, the immediate biochemical precursor of progesterone, however, was inactive, and this suggested to them a role of vitamin A in the conversion of pregnenolone to progesterone This experiment is analogous to our experiment in which DOC was inactive in the A deficient animal, while cortisone was active in glyconeogenesis

Because of this report, we have examined progesterone biosynthesis from cholesterol (see Tables XVIII, XIX, and XXII) Recently Halkerston *et al* (1959) reported that the conversion of cholesterol to progesterone was accomplished by a TPNH requiring enzyme found in the adrenal mitochondria The work was repeated using deficient and normal rat adrenal mitochondria (Table XXII) Nonradioactive pregnenolone was added in

TABLE XXIII

SEVERE VITAMIN A DEFICIENCY AND CHOLESTEROL SYNTHESIS

Vitamin A treatment	Treatment	% Incorporated into unsaponifiable fraction	Incorp into digitonin ppt /Incorp into unsap) × 100	Sp act of digitonin ppt measured as cholesterol (c p m /mg)
−	Complete system	38 0	12 8	32 404
−	Complete system	28 4	17 4	32 924
−	Complete system + G 1 P[b]	28 2	15 9	29 842
−	Complete system + G 1 P	20 1	27 2	36 510
+	Complete system	25 3	44 0	74 182
+	Complete system	21 5	26 8	38 389
+	Complete system + G 1 P	22 8	42 2	64 150
+	Complete system + G 1 P	31 8	43 6	92 377

Complete system contained 3 ml of 10 000 g supernatant (pH 7 4) ATP 0 7 mM DPN 0 5 mM TPN 0 4 mM and 2 μc of mevalonic C^{14} acid 0 23 mM

[b] Glucose 1 phosphate 8 mM

some cases in an effort to study the conversion of the intermediate pregnenolone to progesterone The data presented in these tables show clearly that in a severely deficient animal (Table XIX) progesterone biosynthesis is depressed but that in the more mildly deficient animal (Table XXII) (weight plateaued, but no eye lesions) there is no block in progesterone biosynthesis nor in the conversion of pregnenolone to progesterone even though there is still a depression in corticosterone synthesis

Hays and Kendall (1954 1956) who have reported the fact that progesterone will maintain pregnancy in vitamin A deficient animals (rabbits) have recently shown that this effect is not specific for vitamin A and they suggest that progesterone is related to the mobilization of nutrients from the body stores (Kendall and Hays 1960)

c Cholesterol Biosynthesis Gloor and Wiss (1959) have reported that in

the intact animal vitamin A deficiency causes a lowering in the incorporation of mevalonic acid C^{14} into cholesterol Since this was in contrast to our earlier finding *in vivo* and *in vitro* of no effect of vitamin deficiency on the incorporation of acetate into cholesterol (Wolf *et al*, 1957b), we have repeated this work, using both acetate and mevalonic acid as cholesterol precursors, and have found again no effect of vitamin A deficiency on the labeling of cholesterol from acetate At the same time, the inhibition of

TABLE XXIV
MILD VITAMIN A DEFICIENCY AND CHOLESTEROL SYNTHESIS

Vitamin A treatment	Treatment	% Incorporated into unsaponifiable fraction	(Incorp into digitonin ppt /Incorp into unsap) × 100	Sp act of digitonin ppt measured as cholesterol (c p m /mg)
−	Complete system[a]	28 0	69 7	21 433
−	Complete system	29 7	74 9	24 454
−	Complete system + G 6 P[b]	27 5	71 3	21 572
−	Complete system + G 6 P	21 4	73 0	17,200
−	Complete system + G 6 P + G 6 PDH	30 6	61 2	20 585
−	Complete system + G 6 P + G 6 PDH	23 2	62 3	15,877
+	Complete system	35 2	56 4	21 867
+	Complete system	36 2	51 9	20 641
+	Complete system + G 6 P	27 9	54 7	16,760
+	Complete system + G 6 P	36 1	62 1	24 654
+	Complete system + G 6 P + G 6 PDH	37 3	47 0	19 667
+	Complete system + G 6 P + G 6 PDH	25 3	48 6	13 520

[a] Complete system contained 3 ml of 10 000 *g* supernatant (pH 7 4) ATP 0 7 m*M* DPN 0 5 m*M* TPN 0 4 m*M* and 0 33 μc of mevalonic 2 C^{14} acid 0 23 m*M*
[b] Glucose 6 phosphate 8 m*M*
Glucose 6 phosphate dehydrogenase ? units

incorporation of mevalonic acid C^{14} into cholesterol reported by Gloor and Wiss (1959) *in vivo* was found *in vitro* The data for this experiment are given in Table XXIII The animals used in this experiment are the same severely deficient animals as were used in the corticosterone experiment reported in Tables XX and XXI

Since this again might be due to the general debility of a severe deficiency, this experiment was repeated, using the more mildly deficient animals of Table XXII In this case, as can be seen from the data in Table XXIV, no effect of the vitamin A deficiency on the conversion of mevalonic acid to

cholesterol was found even though these animals had been proved deficient enough to show a reduction in corticosterone biosynthesis (Wright, 1960)

6 *Effect of the* in Vitro *Addition of Vitamin A*

The *in vitro* addition of vitamin A and vitamin A acid to adrenal homogenates from normal and from vitamin A deficient rats has shown a stimulating effect on corticosterone biosynthesis in the deficient adrenal as can be

TABLE XXV

EFFECT OF THE *In Vitro* ADDITION OF VITAMIN A ACID ON CORTICOSTERONE BIOSYNTHESIS

Incubation addition	C^{14} activity in corticosterone in deficient tissue (d p m)	C^{14} activity in corticosterone in normal tissue (d p m)
Homogenate	2862	14 086
Homogenate + vitamin A acid	7641	13 975

TABLE XXVI

EFFECT OF THE *In Vitro* ADDITION OF VITAMIN A ON THE CONVERSION OF CHOLESTEROL TO CORTICOSTERONE

Incubation addition	C^{14} Activity in corticosterone in deficient tissue (d p m)	C^{14} Activity in corticosterone in normal tissue (d p m)
Whole homogenate	9630	13 706
Whole homogenate + vitamin A alcohol[b]	14 998	—
Whole homogenate + vitamin A acid	16 576	—[c]

DPN and TPN 0 5 m*M* ATP 1 m*M* 1 μc cholesterol 4 C^{14} was added to each incubation Incubated for 1 hour under 95 % O_2 5 % CO_2 at 37 C Total volume was 3 ml

[b] Vitamin A alcohol and vitamin A acid added at levels of 1 3 μmoles per incubation

[c] Not determined

seen from the data given in Tables XXV and XXVI (Van Dyke *et al*, 1960b) This would indicate still more clearly that vitamin A functions in some direct way in glucocorticoid biosynthesis This, however, will not be definitely established until a specific enzyme, e g , 11 β hydroxylase, has been isolated and dissociated into its component enzyme and cofactor parts The work of Tompkins *et al* (1958) and of Ober (1958) indicates that with continued fractionation of the 11 β hydroxylase enzyme which converts DOC to corticosterone, enzyme activity is lost This could possibly indicate a loss or destruction of a vitamin A cofactor required by this enzyme

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−	Complete system	29 7	74 9	24,454
−	Complete system + G 6 P[b]	27 5	71 3	21 572
−	*Complete system + G 6 P*	*21 4*	*73 0*	*17 200*
−	Complete system + G 6 P + G 6 PDH	30 6	61 2	20 585
−	Complete system + G 6 P + C 6 PDH	23 2	62 3	15,877
+	Complete system	35 2	56 4	21 867
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[a] Complete system contained 3 ml of 10 000 *g* supernatant (pH 7 4) ATP 0 7 mM DPN 0 5 mM TPN 0 4 *mM and* 0 33 *μc of mevalonic* 2 C^{14} acid 0 23 *mM*

[b] Glucose-6 phosphate 8 mM

[c] Glucose 6 phosphate dehydrogenase 2 units

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−	Complete system[a]	28.0	69.7	21,433
−	Complete system	29.7	74.9	24,454
−	Complete system + G-6-P[b]	27.5	71.3	21,572
−	Complete system + G-6-P	21.4	73.0	17,200
−	Complete system + G-6-P + G-6-PDH	30.6	61.2	20,585
−	Complete system + G-6-P + G-6-PDH	23.2	62.3	15,877
+	Complete system	35.2	56.4	21,867
+	Complete system	36.2	51.9	20,641
+	Complete system + G-6-P	27.9	54.7	16,760
+	Complete system + G-6-P	36.1	62.1	24,654
+	Complete system + G-6-P + G-6-PDH	37.3	47.9	19,667
+	Complete system + G-6-P + G-6-PDH	25.3	48.6	13,520

[a] Complete system contained 3 ml. of 10,000 *g* supernatant (pH 7.4), ATP 0.7 m*M*, DPN 0.5 m*M*, TPN 0.4 m*M*, and 0.33 μc. of mevalonic-2-C^{14} acid 0.23 m*M*.

[b] Glucose-6-phosphate 8 m*M*.

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IV Conclusions (Summary)

Experiments to elucidate a function of vitamin A in the animal body have indicated one site of action as involving glycogen biosynthesis in the intact animal from such precursors as acetate, lactate, and glycerol

The effect of vitamin A deficiency on glycogen biosynthesis from acetate in the animal body was prevented by the administration of glucocorticoid hormone but not by ACTH and was interpreted as indicating a chemical adrenalectomy with regard to glucocorticoid biosynthesis

Whereas cortisone was found to return glycogen biosynthesis from acetate to normal, deoxycorticosterone was inactive, indicating a possible block in their interconversion in vitamin A deficiency

Total adrenal steroid production was found to be lowered in vitamin A deficiency, and this steroid production was returned to normal within 4 hours after vitamin A administration to deficient animals

In *in vitro* studies it was found that glucose 6 phosphate, TPNH, or glycogen would increase total steroid production, particularly in the vita min A deficient adrenal, however, the levels of glucose 6 phosphate dehy drogenase, hexokinase, phosphorylase, and transhydrogenase in the liver were unaffected by vitamin A deficiency

Direct study of the pathway of glucocorticoid biosynthesis in rat adrenal tissue showed that, in the severely vitamin A deficient animal many steps in the synthetic pathway appear to be inhibited, including the steps meva lonic acid to cholesterol, cholesterol to progesterone (possibly pregnenolone to progesterone) cholesterol to deoxycorticosterone, and deoxycorticoster one to corticosterone On the other hand, 17 hydroxydeoxycorticosterone accumulates in the deficient adrenal

In less severely deficient animals only the step deoxycorticosterone to corticosterone appears to be inhibited

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Experiments to elucidate a function of vitamin A in the animal body have indicated one site of action as involving glycogen biosynthesis in the intact animal from such precursors as acetate, lactate, and glycerol

The effect of vitamin A deficiency on glycogen biosynthesis from acetate in the animal body was prevented by the administration of glucocorticoid hormone but not by ACTH and was interpreted as indicating a chemical adrenalectomy with regard to glucocorticoid biosynthesis

Whereas cortisone was found to return glycogen biosynthesis from acetate to normal, deoxycorticosterone was inactive, indicating a possible block in their interconversion in vitamin A deficiency

Total adrenal steroid production was found to be lowered in vitamin A deficiency, and this steroid production was returned to normal within 4 hours after vitamin A administration to deficient animals

In *in vitro* studies it was found that glucose 6 phosphate, TPNH, or glycogen would increase total steroid production, particularly in the vitamin A deficient adrenal, however, the levels of glucose 6 phosphate dehydrogenase, hexokinase, phosphorylase, and transhydrogenase in the liver were unaffected by vitamin A deficiency

Direct study of the pathway of glucocorticoid biosynthesis in rat adrenal tissue showed that, in the severely vitamin A deficient animal many steps in the synthetic pathway appear to be inhibited, including the steps mevalonic acid to cholesterol, cholesterol to progesterone (possibly pregnenolone to progesterone), cholesterol to deoxycorticosterone, and deoxycorticosterone to corticosterone On the other hand, 17 hydroxydeoxycorticosterone accumulates in the deficient adrenal

In less severely deficient animals only the step deoxycorticosterone to corticosterone appears to be inhibited

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Vitamin A and Lipid Metabolism

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I Introduction

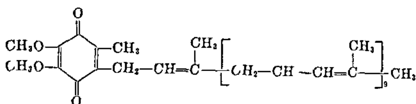
The main aspects of the function of vitamin A in metabolism have been reviewed by Lowe and Morton (1956) in their excellent and comprehensive article in Volume XIV of *Vitamins and Hormones*

Since then the problem of the functional role of vitamin A in steroid metabolism already indicated in that review, has been developed further Part of this problem, that concerning vitamin A and steroid hormones, is discussed by Johnson and Wolf (1960) whereas this article will mainly deal with the function of vitamin A in cholesterol and ubiquinone biosynthesis

II The Ubiquinones (Coenzymes Q)

Lowe *et al* (1953) observed that the livers of vitamin A deficient rats were greatly enriched with two unknown substances named SA and SC and characterized by ultraviolet spectra They were found to be widely distributed in organs of different animals (Morton, 1955) The first hypothesis assuming the substance SA to have an unsaturated steroid structure was abandoned, a dimethoxymethylbenzoquinone with a long isoprenoid side chain was postulated, and the name ubiquinone was proposed (Fahmy *et al* 1958) and Morton *et al* 1958a)

The definite structure determination was performed with a ubiquinone isolated from pig heart having almost the same properties as the ubiquinone from rat liver (Morton *et al*, 1958b, Morton 1958 and Lester *et al*, 1958)



(I)

It turned out to be a substituted benzoquinone with a side chain of 50 carbon atoms (I)

Isoprenologs of ubiquinone (50) with side chains of 45, 40, 35, and 30 carbon atoms, i e with 9, 8, 7, and 6 isoprene units, were isolated from different microorganisms (Lester and Crane, 1959, Gloor *et al*, 1958) The first ubiquinone isolated from vitamin A deficient rat liver turned out to be a mixture of about 80% ubiquinone (45) and 20% ubiquinone (50) (Gloor and Wiss, 1959a) The identifications were made by melting points, mixed melting points, X ray powder diagrams using synthetic ubiquinone (45) and (50) as references and by paper chromatograms according to Lester and Ramasarma (1959) and Ruegg *et al* (1959) In addition minor amounts of ubiquinone (40) and (35) were found in rat liver (Lawson *et al*, 1960)

III Biosynthesis of Ubiquinones by Rat

The ubiquinones consist of a quinone derivative with a long isoprenoid side chain and are quite similar in these respects to vitamins E, K_1, and especially to vitamin K_2 One is inclined therefore to assume that the ubiquinones cannot be synthesized by the higher animal (Fig 1) However, after administration of 2 C^{14} mevalonic acid to rats, in addition to cholesterol and squalene, ubiquinone was found to be labeled (Gloor and Wiss, 1958) The upper part of Fig 5 shows the distribution of the radioactivity in the different fractions of the unsaponifiable material of rat liver

As indicated in the upper part of Fig 5, in the first experiments on the incorporation of radioactivity of labeled mevalonic acid into the ubiquinones of rat liver, alumina chromatography was used for the separation of the ubiquinones from other substances of the unsaponifiable material A separation of ubiquinone (45) and (50) was not achieved by this procedure It became therefore necessary to reinvestigate this point By separation of a ubiquinone mixture, biosynthetically labeled after administering 2 C^{14} mevalonic acid it became clear that both ubiquinone (45) and (50) were radioactive (Fig 2) The distribution of the radioactivity between these two corresponds well with the amount of substance, thus indicating that both labeled ubiquinones have about the same specific activity (Gloor and Wiss, 1960)

The investigations of Dialameh and Olson (1959), Olson and Dialameh

(1960), and Olson *et al* (1960) indicated that ubiquinone (50) was synthesized mainly by the intestinal flora when C^{14} acetate was used as a precursor Our experiments in which the same amount of 2 C^{14} mevalonic acid was administered orally and parenterally do not support this possibility Table I shows clearly that no difference in the distribution of the radio

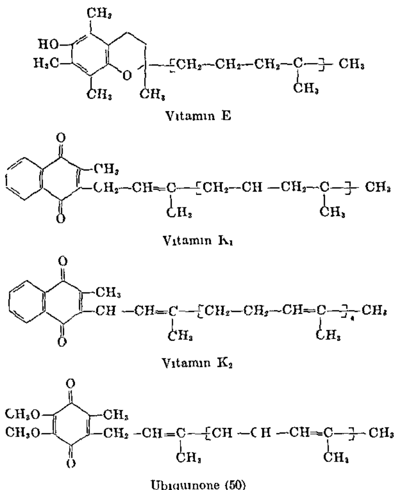
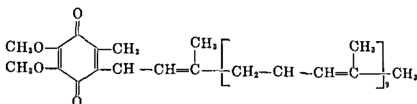


FIG 1 Structural relationship between vitamins E K₁ K and the ubiquinones

activity between ubiquinone (45) and (50) could be detected, when the results after oral and parenteral dosing with C^{14} mevalonic acid are compared

Our knowledge of biosynthetic reactions occurring in higher animals makes it probable that in ubiquinones mevalonic acid is used for the formation of the side chain only Aromatic and benzoquinone compounds are generally essential nutrients for these animals A definite proof of this suggestion was possible by chemical degradation of biosynthetically labeled



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It turned out to be a substituted benzoquinone with a side chain of 50 car bon atoms (I)

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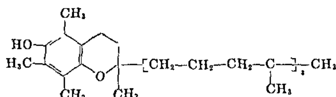
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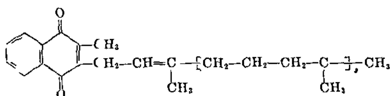
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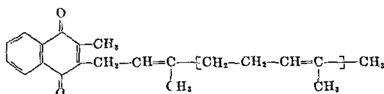
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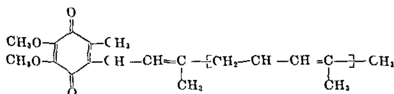
Vitamin E



Vitamin K_1



Vitamin K_2



Ubiquinone (50)

FIG 1 Structural relationship between vitamins E K_1 K_2 and the ubiquinones

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ubiquinone mixtures isolated from rat liver after dosing with 2 C^{14} mevalonic acid After ozonolysis, acetone and levulinaldehyde were obtained as radioactive dinitrophenylhydrazones (Wiss, 1959, Lawson *et al*, 1960) In a further experiment, using 60 mg of biosynthetically labeled ubiqui

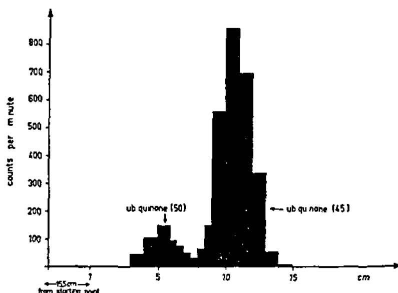


FIG 2 Paper chromatogram from rat liver after dosing with 2 C^{14} mevalonic acid Ubiquinone (50) 540 c p m = 16% ubiquinone (45) 2760 c p m = 84% Whatman 3 MM impregnated with Dow Corning silicone fluid no 550 solvent *n* propanol water (4 1)

TABLE I

BIOSYNTHESIS OF UBIQUINONE (45) AND UBIQUINONE (50) AFTER DOSING RATS WITH 2 C^{14} MEVALONIC ACID

Animal status	Application	Ubiquinones isolated (mg)	Radioactivity in ubiquinones after paper chromatography			
			c p m found in		% Radioactivity found in	
			Ubiquinone (45)	Ubiquinone (50)	Ubiquinone (45)	Ubiquinone (50)
Normal	Intraperitoneal	1 98	14 710	6 720	69	31
Vitamin A deficient	Intraperitoneal	1 91	15 010	6 140	71	29
Vitamin A deficient	Oral	2 05	15 100	5 110	75	25

Three animals per group 0 5 mg per animal 40 × 10^6 c p m administered per group For comparison about 8% of the applied radioactivity was found in the unsaponifiable material about 4% thereof as ubiquinones

It was considered that solanesol phosphate, and pyrophosphate, might be the real precursors, and that these esters might not be split by the alkali saponification procedure used In this case solanesol phosphate or pyro phosphate would not be found by the method used In a second extraction procedure this possibility was taken into account and after alkali saponifica tion the water layer was acidified, again made alkaline, and extracted a second time But even by this method no indication of the existence of radioactive solanesyl ester could be obtained The results of these experi ments, which are summarized in Fig 4, are, however, not sufficient to ex clude solanesol or a solanesyl ester as possible precursors of the ubiquinones, because such precursors need not necessarily be present in measurable amounts in tissues *in vivo*

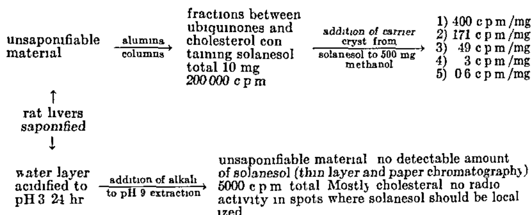


FIG 4 Solanesol from rat livers after feeding 2 C^{14} mevalonic acid

As mentioned earlier 2 C^{14} mevalonic acid proved to be a precursor of the side chain only of ubiquinones (45) and (50) Therefore a number of labeled substances considered as possible precursors of the benzoquinone part of the molecule were administered to rats and their incorporation into the ubiquinones was tested (Table II) After feeding randomly labeled C^{14} phenylalanine, a very low activity could be detected in the unsaponifiable fraction of the liver and in the ubiquinone (Olsen, 1960, Wiss and Gloor, 1960) In our opinion no definite conclusion can be drawn from these results, because the incorporation into the ubiquinone may have been due to ace tate, formed by metabolic degradation of the phenylalanine entering into the side chain The low incorporation ratio and the observation that radio activity was found also in the cholesterol, favors this possibility DL-α Tocopherol (Gloor and Wiss, 1959b, Alaupovic and Johnson, 1959), ubiq uinone (30) (Rudney, 1960, Wiss and Gloor, 1960), and plastoquinone (Wiss and Gloor, 1960) could be excluded as possible precursors In the light of the findings of Martius and Esser (1958) that methylnaphthoqui

none is used for the synthesis of vitamin K_2 isoprenologs in the animal, the 2,3-dimethoxy 5 methyl 1,4 benzoquinone was of special interest No significant incorporation of this tritium labeled substance could be detected (Gloor and Wiss, 1960)

An interesting observation is reported by Rudney (1960) and Rudney and Sugimura (1960), who found that labeled formate is not used in an appreciable amount for synthesizing the methyl of the methoxy groups of

TABLE II

DIFFERENT POSSIBLE PRECURSORS OF UBIQUINONE

Substance administered	Nutritional status of rats	Dose (c p m)	Radioactivity in the unsaponifiable material (c p m)	% of dose in unsaponifiable material	% of dose in ubiquinone (45)
2 C Mevalonic acid	Normal	14 × 10	150 000	11	0 5
2 C Mevalonic acid	Vit A deficient	14 × 10⁶	120 000	9	1 5
Phenylalanine randomly labeled + 5 mg mevalonic acid	Normal	175 × 10	1 000	0 001	Not investigated
Phenylalanine randomly labeled + 5 mg mevalonic acid	Vit A deficient	175 × 10⁶	1 300	0 001	Not investigated
2 3 Dimethoxy 5 methyl 1 4 benzoquinone (tritium)	Vit A depleted	500 × 10	1 380 000	0 055	Activity lost after carrier crystallization
C^{14}-D Glucose	Vit A depleted	300 × 10⁶	6 000	0 00	Trace
DL-α Tocopherolacetate (tritium)	Normal	45 × 10	11 400 000	2 4	3 Different compounds but nothing in ubiquinone
Ubiquinone (30) (tritium)	Vit A-depleted	95 × 10	800 000	8 5	No activity in ubiquinone (45)
Plastoquinone (tritium)	Normal	138 × 10⁶	48 000	0 35	Starting material destroyed by alkali no activity in ubiquinone (45)

the ubiquinones by the rat This favors the theory that a precursor very similar to the ring system of the ubiquinones is available to the rat from food or from the intestinal flora

IV THE INFLUENCE OF VITAMIN A DEFICIENCY ON CHOLESTEROL, UBIQUINONE AND SQUALENE BIOSYNTHESIS IN THE RAT

The influence of vitamin A deficiency on the biosynthesis of the ubiquinones from C^{14} mevalonic acid was investigated with respect to the observation of Lowe *et al* (1953) that the ubiquinone content of the liver of vitamin A deficient rats was much higher than that of normal animals The question has to be answered whether this higher level of ubiquinone is only

a symptom of fatty liver or other disorders occurring in vitamin A deficiency, or whether it must be attributed to an enhanced ubiquinone biosynthesis. In the experiment reported in Fig. 5, the incorporation of C^{14} mevalonic acid into liver ubiquinones was about six times as high in vitamin A-deficient, as in normal rats. There is only a small difference between the total radioactivity of the unsaponifiable material of normal and vitamin A-deficient rats. Its distribution between cholesterol and squalene, however, was found to be greatly changed the proportion in cholesterol being reduced from 95% to about 50%, and that in squalene enhanced from 2%

TABLE III

SYNTHESIS OF SQUALENE, CHOLESTEROL, AND UBIQUINONES AFTER DOSING RATS WITH 2-C^{14} MEVALONIC ACID DURING INCREASING VITAMIN A DEFICIENCY

Days of vitamin A depletion	Rat weights in gm duplicates		Total radioactivity in unsaponifiable material of the liver (c.p.m.)	% Radioactivity in different fractions radioactivity of the *unsaponifiable taken as 100%*		
				Squalene	Ubiquinones	Cholesterol
0	37	35	98 500	2.0	2.5	95.5
7	59	53	99 000	8.7	10.7	80.6
14	87	79	101 800	19.3	12.8	67.9
21	101	106	67 400	25.0	16.1	58.9
24	90	74	63 500	26.9	18.5	54.6
Single dose of 500 I.U. vitamin A orally per animal						
20 Hours after vitamin A dose	105	85	95 500	13.8	10.3	75.9
7 Days after vitamin A dose	131	101	83 400	11.7	8.7	79.6

to about 40%. As mentioned before, the ubiquinones of normal rat liver turned out to be a mixture mainly consisting of ubiquinone (45) and (50). Both were found labeled after giving 2-C^{14} mevalonic acid to normal rats. Table I shows that the incorporation ratio for both these ubiquinones is about the same in vitamin A deficient as in normal rats, thus indicating that the synthesis of both is enhanced. The influence of vitamin A deficiency on C^{14} mevalonic acid incorporation was tested in the course of the depletion period. Table III shows that an effect can be seen very early and that by giving vitamin A to deficient animals, a clear trend to normal values, even after a few hours, can be observed. This indicates that these metabolic alterations are not a secondary effect, due for instance to tissue damage, but that vitamin A is acting at a metabolic level. This suggestion

is supported by *in vitro* experiments The incorporation of C^{14} mevalonic acid into squalene and cholesterol was proved using rat liver homogenates Table IV shows that in vitamin A deficiency the incorporation of C^{14} mevalonic acid into squalene is clearly enhanced, at the expense of cholesterol (Weber *et al*, 1960) *In vitro* no ubiquinone synthesis could be found This

TABLE IV

INCORPORATION OF 2 C^{14} MEVALONIC ACID INTO SQUALENE AND CHOLESTEROL (LIVER HOMOGENATE)

Rat livers	Number of rats	Incorporated radio activity into unsaponifiable material	Distribution of radio activity	
			Squalene	Cholesterol
Normal (pair fed controls)	12	15 2%	12 6%	81 4%
Vitamin A deficient	12	8 3%	50 7%	45 0%
Significance of differences between mean values	—	$p < 0\ 01$	$p < 0\ 01$	$p < 0\ 01$

Test conditions 3 ml of 28% homogenate in buffer pH 7 4 400 μg of 2 C^{14} mevalonic acid (14 2 × 10^6 c p m) each 2 mg of ATP and DPN final volume 3 8 ml incubation at 38 C for 1 hour under 100% O in a reciprocating water bath

TABLE V

INCORPORATION OF 2 C^{14} MEVALONIC ACID INTO SQUALENE CHOLESTEROL AND UBIQUINONE (LIVER HOMOGENATES)

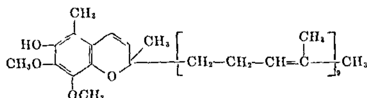
Compound	10 6 × 10^6 c p m of 2 C^{14} mevalonic acid added (incorporated radioactivity in c p m)		
	Incubated under O_2		*In air*
Squalene	52 700	44 300	176 900
Cholesterol	807 300	862 100	539 700
Ubiquinone	<300	<650	<130

Test conditions 3 ml of 28% homogenate in buffer pH 7 4 each 2 mg of ATP and DPN 1 mg of TPNH final volume 3 8 ml Incubation at 38 C for 3 hours in a reciprocating water bath

favors the suggestion already made that a special precursor, yielding the benzoquinone part of ubiquinone is needed (Table V)

V UBICHROMENOL

Substance SC first reported by Heaton *et al* (1955), present in much smaller amounts than ubiquinones in kidney and other tissue of many species, has now been isolated in pure form It is an isomer of ubiquinone (50), having structure (II) (Heaton *et al*, 1957, Laidman *et al*, 1960)



(II)

It seems obvious that ubichromenol is closely related to ubiquinone (50) and that they can eventually be converted into each other Further studies, however, are necessary to find out whether this reaction is an enzymatic or spontaneous one (Links, 1960) By the structural relationship of these two substances, the previous observation that ubichromenol, in addition to ubiq uinone, is enriched in vitamin A deficient rats, finds a reasonable expla nation

VI Discussion

Morton and his group, even before having elucidated the structure of SA (ubiquinone) and SC (ubichromenol), found interesting relationships be tween these two substances and vitamin A deficiency in the rat They sug gested that SA and SC are metabolically related to cholesterol, in that they are metabolic intermediates that accumulate in the deficiency state (Heaton *et al*, 1957 Green *et al*, 1957a) As it was known that SA and SC are highly unsaturated compounds, an oxidation or reduction reaction was thought to be involved in the vitamin A dependent metabolic reaction

The rise in SA or SC which occurs in vitamin A deficient rat tissue did not appear in the chicken (Lowe *et al*, 1957)

Contrary to the clear influence of vitamin A deficiency on the ubiqui none and ubichromenol content of rat liver, the cholesterol content of blood and liver was found unchanged (Green *et al* 1957b, Smith 1934) Thus an earlier observation of Ralli and Waterhouse (1933) could not be con firmed On the other hand it could be shown that a high cholesterol intake depressed the liver storage of vitamin A in rats and cockerels (Katingar, 1952 Green *et al*, 1957a)

The first studies of the influence of vitamin A deficiency on the biosyn thesis of cholesterol have been reported by Wolf *et al* (1957) With labeled C^{14} acetate as precursor no definite influence of vitamin A deficiency could be detected Results obtained after administration of labeled C^{14} mevalonic acid have shown a reduction of the incorporation into cholesterol in vitamin A deficient rats (Gloor and Wiss 1959b) These results need not necessarily be conflicting The difference may be explained by assuming that only on the addition of an appreciable amount of an immediate precursor of choles terol (i e mevalonic acid) does the enzymatic reaction in which the vitamin A may be involved, become limiting An alternative explanation may be

is supported by *in vitro* experiments The incorporation of C^{14} mevalonic acid into squalene and cholesterol was proved using rat liver homogenates Table IV shows that in vitamin A deficiency the incorporation of C^{14} mevalonic acid into squalene is clearly *enhanced, at the expense of cholesterol* (Weber *et al* , 1960) *In vitro* no ubiquinone synthesis could be found This

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Normal (pair fed controls)	12	15 2%	12 6%	81 4%
Vitamin A deficient	12	8 3%	50 7%	45 0%
Significance of differences between mean values	—	$p < 0 01$	$p < 0 01$	$p < 0 01$

[a] Test conditions 3 ml of 28% homogenate in buffer pH 7 4 400 μg of 2 C^{14} mevalonic acid (14 ° X 10^6 c p m) each 2 mg of ATP and DPN final volume 3 8 ml incubation at 38 C for 1 hour under 100% O in a reciprocating water bath

TABLE V

INCORPORATION OF 2 C^{14} MEVALONIC ACID INTO SQUALENE CHOLESTEROL AND UBIQUINONE (LIVER HOMOGENATES)[a]

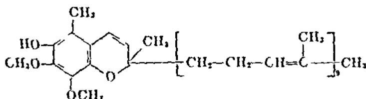
Compound	10 6 X 10^6 c p m of 2 C^{14} mevalonic acid added (incorporated radioactivity in c p m)		
	Incubated under O_2		In air
Squalene	52,700	44 300	176,900
Cholesterol	807,[illegible]00	862 100	539 700
Ubiquinone	<300	<650	<130

[a] Test conditions 3 ml of ?8% homogenate in buffer pH 7 4 each ? mg of ATP and DPN 1 mg of TPNH final volume 3 8 ml Incubation at 38 C for 3 hours in a reciprocating water bath

favors the suggestion already made that a special precursor, yielding the benzoquinone part of ubiquinone is needed (Table V)

V UBICHROMENOL

Substance SC first reported by Heaton *et al* (1955), present in much smaller amounts than ubiquinones in kidney and other tissue of many species, has now been isolated in pure form It is an isomer of ubiquinone (50), having structure (II) (Heaton *et al* , 1957, Laidman *et al* , 1960)



(II)

It seems obvious that ubichromenol is closely related to ubiquinone (50) and that they can eventually be converted into each other Further studies however, are necessary to find out whether this reaction is an enzymatic or spontaneous one (Links, 1960) By the structural relationship of these two substances, the previous observation that ubichromenol in addition to ubiquinone, is enriched in vitamin A-deficient rats, finds a reasonable explanation

VI Discussion

Morton and his group, even before having elucidated the structure of SA (ubiquinone) and SC (ubichromenol), found interesting relationships between these two substances and vitamin A deficiency in the rat They suggested that SA and SC are metabolically related to cholesterol, in that they are metabolic intermediates that accumulate in the deficiency state (Heaton *et al*, 1957, Green *et al*, 1957a) As it was known that SA and SC are highly unsaturated compounds, an oxidation or reduction reaction was thought to be involved in the vitamin A dependent metabolic reaction

The rise in SA or SC which occurs in vitamin A deficient rat tissue did not appear in the chicken (Lowe *et al* 1957)

Contrary to the clear influence of vitamin A deficiency on the ubiquinone and ubichromenol content of rat liver the cholesterol content of blood and liver was found unchanged (Green *et al* 1957b, Smith 1934) Thus an earlier observation of Ralli and Waterhouse (1933) could not be confirmed On the other hand, it could be shown that a high cholesterol intake depressed the liver storage of vitamin A in rats and cockerels (Katingar, 1952 Green *et al*, 1957a)

The first studies of the influence of vitamin A deficiency on the biosynthesis of cholesterol have been reported by Wolf *et al* (1957) With labeled C^{14} acetate as precursor, no definite influence of vitamin A deficiency could be detected Results obtained after administration of labeled C^{14} mevalonic acid have shown a reduction of the incorporation into cholesterol in vitamin A deficient rats (Gloor and Wiss, 1959b) These results need not necessarily be conflicting The difference may be explained by assuming that only on the addition of an appreciable amount of an immediate precursor of cholesterol (i e mevalonic acid) does the enzymatic reaction in which the vitamin A may be involved, become limiting An alternative explanation may be

that, since only a very small amount of the applied dose of C^{14} acetate is incorporated compared to C^{14} mevalonic acid, the acetate method is insufficiently sensitive to demonstrate the relatively small reduction of cholesterol biosynthesis due to vitamin A deficiency in the rat

The results obtained on the 2 C^{14} mevalonic acid incorporation into cholesterol, squalene, and ubiquinones in normal and vitamin A deficient rats, led to the suggestion that vitamin A is involved in one or more metabolic reactions necessary for the synthesis of cholesterol The reported data, however, do not warrant speculation as to which reaction(s) depend(s) on vitamin A The only possible suggestion is that the interaction of vitamin A takes place in a step following the squalene formation Thus it can be explained that in vitamin A deficiency the incorporation of C^{14} mevalonic acid into cholesterol is reduced, whereas more radioactivity is found in the

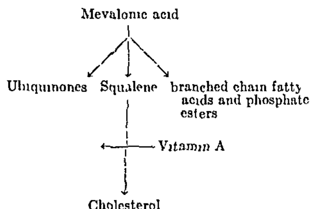


Fig 6 Suggested outline of the interaction of vitamin A in the metabolism of mevalonic acid

squalene fraction The rise in the ubiquinone synthesis may be due to a feedback action The observation that in vitamin A deficiency the radioactivity of the fractions containing the branched chain acids or the phosphate or pyrophosphate esters of isoprenoid alcohols is enhanced, lies in the same line (Gloor and Wiss, 1959a) Figure 6 summarizes these outlined metabolic relationships

VII Summary

The structure of ubiquinones (SA) and ubichromenol (SC) previously found to be enriched in livers of vitamin A deficient rats has been elucidated The ubiquinones widely distributed in natural material are dimethoxymethylbenzoquinone derivatives with long isoprenoid side chains The ubichromenol turned out to be the corresponding chromene derivative, obviously formed by ring closure of the ubiquinone (50)

The rat liver contains a mixture of ubiquinone (45) and (50) The side

chain of both is synthesized by the rat, using mevalonic acid as a precursor

In vitamin A deficiency the incorporation of C^{14} mevalonic acid in both ubiquinones (45) and (50) is enhanced A remarkable rise of the incorporation into squalene is also observed, whereas the biosynthesis of cholesterol is reduced The sum of the total incorporation into these substances is little affected by vitamin A deficiency

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The Pathology of Vitamin A Deficiency

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I Introduction

The pathology of vitamin A deficiency presents a complex picture Thus the lesions sustained vary according to the species and age of the animal and the environmental conditions Sensitivity to the effects of deficiency is also influenced by sex Secondary lesions, arising frequently from infections and sometimes from other causes, are often imposed upon the lesion immediately due to avitaminosis A In contrast to the original injury these secondary effects may sometimes fail to respond to treatment with vitamin A As a further complication diseases not originating from vitamin A deficiency may affect the vitamin A status sometimes to an extent sufficient to cause an "induced" or conditioned" deficiency of the vitamin Work on experimental animals suggests that a complete deficiency of vitamin A from the body, unlike deficiencies of some other fat soluble vitamins invariably fatal The large stores of vitamin A which can be accum in times of dietary plenty, however can postpone the effects of subs deprivation of the vitamin for long periods

It would be a hopeless task, in a short review, to attempt to describe systematically all the effects of avitaminosis A in different animals, with a complete list of references to the original investigations on which our information is based We must be content, therefore, with an account of the more important aspects of the pathology of vitamin A deficiency in broad outline Points of detail will be mentioned only when they seem to give rise to more general implications

As a preliminary to our discourse a few words on terminology may be helpful "Primary" deficiency of vitamin A will be so described when the diet fails to supply sufficient vitamin A or provitamins to maintain the health of the animal Sometimes the deficiency may be complete and sometimes partial, and the ill effects on the animal may be either acute or chronic These ill effects, moreover, may be more or less specific to avitaminosis A, such as hemeralopia and xerophthalmia or may be nonspecific diseases, such as pneumonia or pyelitis On the other hand a "conditioned," "induced," or "secondary" deficiency of vitamin A will be so described when the diet contains adequate amounts of vitamin A or carotene, but the animal is unable to absorb or metabolize these supplies The term "secondary" will not be used when deficiency of vitamin A merely accompanies a more severe deficiency of some other nutrient, with no evidence of a metabolic or pathological interrelationship between the two deficiencies In these circumstances a description such as "concomitant" or "simultaneous" would seem appropriate in referring to the deficiency of vitamin A, or we might speak of a 'combined" deficiency of the two nutrients

We must remember, however, that it may sometimes be difficult to reconcile convenience in terminology with a strict regard for truth, particularly in relation to forms of deficiency which would generally be regarded as "primary " Thus in the classic examples of vitamin A deficiency in human infants reported by Bloch (1921), the development of xerophthalmia often followed a period of ill health, with debility, diarrhea, and infection To this extent, therefore, the lesions could be described as secondary Even hemeralopia might be regarded, in some instances as a secondary effect of excessive exposure of the retina to bright light It seems a reasonable compromise to classify forms of vitamin A deficiency that are related to low dietary intakes of the vitamin as "primary," even when specific lesions characteristic of avitaminosis A are precipitated or intensified by some different etiological factor

It will be convenient first to review the effects of avitaminosis A as observed under controlled conditions in experimental animals We can then proceed to an account of outbreaks of avitaminosis A that have appeared spontaneously in farm animals and in human subjects

II Experimental Vitamin A Deficiency

1 Different Types of Lesions

At least three basic lesions, or types of lesion, occur in avitaminosis A (1) Lack of vitamin A, required in the form of its aldehyde for the formation of rhodopsin, causes defective dark adaptation Unless complications have ensued this lesion may be regarded as "biochemical" rather than "structural" Thus the response to treatment with vitamin A is rapid and dramatic Since the protein opsin has been found only in the retina, and since vitamin A aldehyde is found only rarely in other sites, we may perhaps look upon the rhodopsin system as a highly specialized mechanism, small but important, which does not typify the behavior of the much larger amounts of vitamin A that are present in the rest of the body Detailed discussion of the effect of vitamin A deficiency on the rhodopsin system can doubtless be left to Professor Wald (2) Lack of vitamin A causes xerosis or keratinization of membranes in many parts of the body The well known xerophthalmia belongs to this group of lesions Epithelia tend to become dry and excessively thick and horny Those membranes having a columnar structure, often associated with the secretion of mucus, give place to thick layers of stratified epithelia as found in the outer part of the epidermis The most common secondary effects of avitaminosis A arise from the bacterial infection of the abnormal membranes In some sites such as the urinary bladder the injury to the membrane may sometimes induce calcification with the formation of stones (3) During growth lack of vitamin A can cause defective modeling of the bones As a result the bones are not compact strong and well shaped but cancellous weak, and excessively thick

Lesions of more than a single type are often superimposed, and the possibility of further types cannot be excluded Thus avitaminosis A can give rise to nerve lesions which cannot always be regarded as secondary effects of the bone lesions Another abnormality is an increase in the pressure of spinal fluid sometimes associated with hydrocephalus These lesions might well be regarded as secondary effects of malformation of the skull bones but a rival theory suggests that deficiency of the vitamin causes the choroid plexus to secrete unduly large amounts of fluid A further problem concerns the sequence of pathological changes that give rise to congenital malformations in the offspring of mothers who are different in vitamin A, and the degree of specificity between the malformation and lack of vitamin A

2 Vitamin A Deficiency in the Rat

This animal was used for the classic studies of Wolbach and Howe (1925) on the effect of avitaminosis A in causing keratinization of membranes,

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blindness, increased cerebrospinal fluid pressure, papillary edema and permanent blindness caused by constriction of optic nerve due to malformed bone

Dogs were used in equally famous experiments by Mellanby (1938–39, 1944), who was the first to realize that deficiency of vitamin A can cause malformation of the bones

Rabbits were used by Mellanby (1934) in early experiments on the association between xerophthalmia and lesions of the trigeminal nerve More recently Millen and his colleagues (1953, 1954a, b) observed hydrocephalus in young rabbits whose mothers had been deprived of vitamin A for various periods before mating, or in young who had been deprived of vitamin A after weaning These workers were first attracted by the possibility that fluid might accumulate in the ventricles because of stenosis of the cerebral aqueduct Later they favored the view that vitamin A deficiency caused an excessive secretion of fluid by the choroid plexuses, with distortion of the aqueduct as a secondary effect

Pigs were subjected to experimental vitamin A deficiency by Hughes *et al* (1929) There was no evidence of xerophthalmia, but the animals nevertheless became blind, and developed incoordination and spasms Nerve lesions were detected at autopsy

Ducks were used by Rigdon (1952) who observed nerve lesions which did not appear to be related to abnormalities in the bones In the chicken, injuries to the kidneys cause abnormalities in the metabolism of uric acid (Capper *et al*, 1931, Elvehjem and Neu 1932) Keratinization in the nasal passages of deficient chickens has been studied by Seifried (1930) and Jungherr (1943)

4 Vitamin A Deficiency in Human Volunteers

Experiments on vitamin A deficiency in human subjects, unlike those on laboratory animals, have usually been carried out on adults All workers who have used rats in bioassays of vitamin A will know how important it is to restrict the young animals to their deficient diet before they have had time to accumulate substantial reserves of vitamin A in their livers If this point is overlooked signs of deficiency will not appear within the usual 3–5 weeks, but may be postponed for many months Experiments on human volunteers are therefore carried out under conditions which would seem very unsuitable if applied to rats It is not surprising therefore, that the results of experiments on avitaminosis A in human subjects have been somewhat disappointing in comparison with the time labor, and expense that they have involved

In early experiments by Booher *et al* (1939), five adults were restricted to a diet from which foods containing more than traces of vitamin A were

omitted Dark adaptation was impaired after periods of 16–124 days, with a mean of 47 days No signs of incipient xerophthalmia could be found on examination with a slit lamp Wald and Steven (1939) found that one young man developed defective dark adaptation after restriction to a diet deficient in vitamin A for 34 days, in spite of his having been heavily dosed with the vitamin before the restriction was imposed The defect responded very rapidly to further dosing with vitamin A

In other experiments longer periods of deprivation have been required before dark adaptation has deteriorated In an experiment on five young men who had previously been dosed with vitamin A, Wald *et al* (1942) were able to observe defective dark adaptation in only two instances Brenner and Roberts (1943) dispensed with preliminary dosing, but failed to observe defective dark adaptation in any of six volunteers after periods of 4½ to 7½ months In both these investigations the levels of carotenoids in the blood fell rapidly, but vitamin A remained at the same resting level as had been found before deprivation was commenced

The well known experiment at Sheffield (Hume and Krebs, 1949), which was carried out on more than 20 volunteers, gave further evidence of the difficulty of inducing vitamin A deficiency in healthy adults As expected blood carotenoids fell rapidly to low levels within the first few weeks of the experiments Observations of blood vitamin A over long periods indicated steady falls in most subjects Eventually in four of the volunteers, after 12–18 months, levels of 40 I U per 100 ml of plasma or less were observed, as compared with levels of about 100 I U at the commencement of deprivation In three of the four subjects dark adaptation was defective, and was found to respond favorably to dosing with vitamin A or carotene No other indications of deficiency of vitamin A could be detected We may conclude, therefore, that stringent restriction to a diet deficient in vitamin A for periods of up to 18 months induced only mild deficiency of vitamin A, as judged by functional tests and clinical examination, in about 20% of the volunteers who took part in the investigation

III Vitamin A Deficiency in Animal Husbandry

1 Liability to Avitaminosis A of Different Species

Of the common farm animals, pigs and poultry may be considered the most vulnerable to deficiency of vitamin A Bovines come next in the order of vulnerability Finally sheep and horses seem relatively immune from danger

These relative vulnerabilities are presumably not directly related to species, but to the diets which are usually provided for the animals by the farmer Thus pigs and poultry often have subsisted on diets based

largely on the coarser fractions of wheat and other cereals Unless supplements of vitamin A are provided, outbreaks of avitaminosis A seem inevitable As long as farmers and animal food manufacturers remember this fact, all goes well Forgetfulness, however, brings its penalty of ailing pigs or dying chickens

Bovines are less liable to avitaminosis A because they usually obtain access to pasture for adequate periods Artificial feeding, however, brings the same dangers as for poultry and pigs Sheep are seldom stall fed to the same extent as bovines, and hence are seldom affected by lack of vitamin A There is ample evidence, of course that avitaminosis A can be readily produced in sheep by experimental diets (Guilbert *et al*, 1937) Adequate proof that sheep suffer spontaneously from avitaminosis A under field conditions, however, is still awaited It is also clear that horses are vulnerable to experimental deficiency of vitamin A (Guilbert *et al*, 1940), but again the importance of the deficiency under field conditions is questionable

2 Vitamin A Deficiency in Pigs

The effects of vitamin A deficiency were observed in pigs fed upon diets consisting exclusively of cereal products before the importance of vitamin A for the nutrition of this animal were recognized (Hart and McCollum, 1914) The animals neither developed frank xerophthalmia nor declined greatly in body weight, which are usually the most obvious signs of deficiency in rats Their most striking abnormality was paralysis of the hind legs, associated with nerve injuries Some of the pigs also became blind In Britain serious outbreaks of vitamin A deficiency occurred among pigs about twenty five years ago (Dunlop, 1934, 1935) The diets, such as a mixture of barley meal, middlings, and fish meal were of types that had been recommended by official agricultural advisers Apparently vitamin A deficiency was only avoided more or less by accident when the diet contained yellow maize when the pigs had access to grass or green vegetables, or when the farmer believed in dosing his animals with cod liver oil Recently there have been further sporadic outbreaks of deficiency, but presumably these have been due to neglect on the farmer's part rather than to lack of knowledge of the importance of vitamin A among agricultural advisers

Another type of abnormality of great theoretical interest, has been observed in the young of sows which have received diets inadequate in vitamin A (Hale 1935) These congenital deformities included lack of eyeballs, cleft lips, cleft palates, extra earlike growths and subcutaneous cysts Congenital absence of the eyeballs has been observed recently in Britain by Goodwin and Jennings (1958), who later have been puzzled by the

production of defective litters followed by normal litters by the same sow, without any obvious change in diet Braude and his colleagues (1951) have emphasized the difficulty of producing congenital lesions experimentally in the offspring of sows which have high initial stores of vitamin A Thus a sow which had already produced five litters while receiving a normal diet was able to produce three more normal litters after restriction to a diet deficient in the vitamin In the fourth litter after restriction, however, the colostrum was inadequate in vitamin A, and the piglets were not reared It appears that the production of offspring with congenital deformities lies intermediate between two other forms of abnormality, first, the complete failure of reproduction and secondly, the production of normal young which cannot be reared

3 Vitamin A Deficiency in Bovines

In cattle as in pigs, the effects of vitamin A deficiency were known long before the discovery of the vitamin Thus Connel and Carson (1896) reported the occurrence of "fat sickness," which was characterized by inflammation of the eyes, or total lack of sight The two main causes of avitaminosis A in bovines are (1) the parching of their pastures by prolonged draught and (2) stall feeding with fodder deficient in carotene In temperate countries deficiency can seldom arise from the first of these causes, and danger mainly arises from inferior fodder, and particularly from the inclusion in the diet of excessive amounts of sugar beet pulp It seems probable that the first effect of vitamin A deficiency, under farming conditions, must be night blindness This condition, however, will usually escape detection and the animal will be permanently blind even in daylight before its illness is detected This blindness is not an aftermath of xerophthalmia and keratomalacia, as in the human subject It results from the damage to the optic nerve associated with abnormalities in the growth of bone, as found by Lane Moore and his colleagues (Moore, 1939, Moore and Sykes, 1941, Moore *et al*, 1935) Externally the eyes usually show no more than excessive tear production, roughness of the lids, and sometimes exophthalmos Examination by the ophthalmoscope shows papilledema (Moore *et al*, 1935) Other effects of the deficiency may include roughness of the coat, excessive salivation, edema, and incoordination Cysts are sometimes found in the pituitary glands (Madsen *et al*, 1942) Reproduction may be defective, and the calves may be born blind and partially paralyzed

An interesting point, mentioned by Blakemore and his colleagues (1957) is the long delay that can sometimes occur between the period when the animal is deprived of vitamin A and the onset of blindness Thus blindness may occur suddenly in bovines which are growing rapidly while receiving

an adequate diet, such as fresh green pasture Inquiry into the past history of such animals usually reveals that at some earlier period they had subsisted on a diet made up largely of sugar beet pulp Presumably injury to the bones occurs during this early period, and the damage to the nerves follows after rapid growth has aggravated the anatomical abnormality in the relationship between nerve and bone

In addition to their liability to primary deficiency of vitamin A, there has been evidence from America that bovines can suffer from an interesting secondary form of avitaminosis A, which was given the name "X disease" (Olafson 1947) The young animal fails to thrive, becomes lean and emaciated, and has a thick, rough coat indicative of hyperkeratosis At autopsy similar thickening is found in the linings of the stomach, bile ducts, gall bladder, and other sites These findings were suggestive of a form of vitamin A deficiency but the immediate cause of the abnormality was traced to poisoning by chlorinated naphthalene derivatives These substances were present either in spent motor oil which was used in making food pellets or in wood preservative which had been applied to the pens in which the animals were kept Hansel and his colleagues (1951) found that in calves suffering from experimentally induced X disease the levels of vitamin A in the blood were much lower than in control calves This suggested that the similarity of the lesions in X disease to those seen in avitaminosis A could be explained on the basis of a secondary deficiency induced by the chlorinated naphthalenes Unfortunately even heavy dosing with vitamin A could not completely prevent the injuries caused by chlorinated naphthalenes although such treatment appeared to be beneficial The evidence that X disease is essentially a form of secondary avitaminosis A, therefore, is suggestive rather than conclusive

IV Clinical Vitamin A Deficiency in the Human Subject

1 *Defective Dark Adaptation*

The value of a meal of liver in curing night blindness was known to Egyptian doctors in 1500 B C and also to the famous Greek philosopher Hippocrates We may assume therefore that this manifestation of vitamin A deficiency has been experienced by mankind at least since the dawn of history Early reports in Britain by Snell (1876) concerned children who were brought to him because they were unable to see in the streets at night and who were cured by dosing with cod liver oil Aykroyd (1944) has reported that night blindness has proved troublesome to fishermen in Newfoundland, who subsisted on poor diets They were able to cure themselves by eating livers taken from cod fish or from sea gulls

Studies of efficiency of dark adaptation have been used in dietary sur

veys for detecting partial deficiency in the vitamin Jehgers (1937) claimed that dark adaptation was abnormal in 35% of a large group of American medical students In 12% of the students, moreover, deficiency was also indicated by subjective night blindness, photophobia, or follicular hyper keratosis Maitra and Harris (1937) found subnormal dark adaptation in 20–36% of the pupils in English elementary schools The poor dark adap tation was improved in pupils who were dosed with vitamin A, but not in others who were left undosed

2 Xerophthalmia and Associated Abnormalities

The classic cases of xerophthalmia described by Bloch (1921) occurred in Danish infants who had been fed upon diets containing skimmed milk After the condition had been recognized as an effect of avitaminosis A its prevention presented no difficulty Further cases of xerophthalmia in prosperous, temperate countries have been so rare that is is difficult to quote authentic instances

In some tropical countries, however, xerophthalmia has continued to claim numerous victims and leads to permanent loss of sight Biswas (1941) made an extensive study of xerophthalmia in India, and was saddened by the frequency of patients coming for treatment too late for their sight to be saved Thus out of 215 patients who came to him for treatment, 127 already had advanced keratomalacia This worker recognized six stages in the progress of the ocular effects of avitaminosis A (1) defective dark adaptation, (2) keratinization and the production of Bitot's spots in the conjunctiva, (3) diminished sensitivity in the cornea, (4) degeneration in the cornea, (5) breakdown in the cornea, with inflammatory reaction in the eye, (6) blindness Of the severe cases 70% were patients under 5 years of age

Numerous reports of xerophthalmia have come also from the East Indies, including a recent paper by Oomen (1958) He associated the condition with diets consisting mainly of rice, and with the inadequate feeding of small children According to this worker the following ocular abnormalities are associated with hypovitaminosis A (1) prexerosis usually combined with hemeralopia, (2) xerosis of the conjunctival epithelium (3) Bitot's spots, (4) xerosis of the corneal epithelium, (5) mummification of the cornea, (6) keratomalacia Of these abnormalities Oomen considers that xerosis of the cornea and conjunctiva are the most closely linked with hypovitaminosis A Other factors may enter into the etiology of Bitot's spots and keratomalacia, but these abnormalities appear nevertheless to be closely associated with the others

In Oomen's work much lower levels of vitamin A were found in the blood of patients with xerophthalmia or keratomalacia than in normal

children in the same social class, who in turn had lower values than were found in children of a higher class Thus, for 42 patients with various ocular lesions, a mean of about 13 I U per 100 ml of serum was found, as compared with 68 I U for 38 poor school children, or 146 I U for 24 children of doctors These results are very different from those reported by Roels and his colleagues (1958) from the Belgian Congo These workers took Bitot's spots as their criterion of vitamin A deficiency For 48 men so affected the mean level of vitamin A was 177 I U per 100 ml as compared with 213 I U for 37 men without spots

In comparing the levels of vitamin A found in these two investigations we must remember that values for vitamin A tend to be lower in young children than in adults This factor alone, however, would explain only part of the wide difference Two explanations seem possible First we may question the reliability of Bitot's spots as an indication of vitamin A deficiency This view has been supported by Rodger (1960), who has failed to find low levels of vitamin A in the blood, defective dark adaptation, or benefit from vitamin A therapy in patients with this abnormality Alternatively we may conclude that it is not necessary for depletion of vitamin A to be complete before signs of deficiency are developed Bitot's spots may be caused by various etiological factors which include hypovitaminosis A If vitamin A deficiency is not the only factor involved we may expect to find Bitot's spots in some subjects having high levels of vitamin A in their blood, although there will be a general tendency for the affected subjects to have lower levels This would result in a low range and a lower mean for the affected subjects as found by Roels and his colleagues

The failure of Bitot's spots to respond to vitamin A therapy may possibly be due to the marks, which take the form of small white flecks or bubbles at the outer corners of the eyes being formed by dead tissues As Oomen (1958) has pointed out the sclerotic conjunctiva becomes transparent and moist after doses of vitamin A have been given for a few days, but the cellular debris forming the spot may be unaffected even after weeks of dosing

3 *The Influence of Sex on the Liability to Injury by Avitaminosis A*

Early work by Birnbacher (1928) in Vienna indicated that human males are much more prone than females to hemeralopia The recent work by Oomen (1958) has shown that this sex relationship also applies to xerophthalmia a sign of more advanced vitamin A deficiency Yet further evidence of the sex difference has been found by Roels and his colleagues (1958) in their studies on Bitot's spots

Evidence of the influence of sex on vitamin A, both in the human subject and in the rat, was reviewed by Moore (1957) Recently Leitner and

his colleagues (1960) found that the mean level of vitamin A in a large group of British men was about 20% higher than in women, whereas the mean carotenoid level was about 8% higher in women than in men The higher level of vitamin A in the blood of the male suggests that the rate of expenditure of the vitamin may be higher than in the female Possibly the lower carotenoid levels in the male indicate that the conversion of carotene is more efficient than in the female Both these differences might well be related to the higher average basal metabolic rate in males, which exceeds the rate in females by some 10%

4 Vitamin A and Skin Diseases

The association between xerophthalmia and a dry scaly skin was noticed by de Gouvea (1883) Subsequently avitaminosis A was accepted by most workers as the cause of a roughness of the skin, due to hyperkeratosis of the hair follicles, which is frequently seen in Indian, African, and Chinese subjects Nicholls (1933) observed the condition frequently among the inmates of Ceylonese prisons, and named it "phrynoderma" or "toad skin" Stannus (1945) and others, however, questioned the validity of relationship between "phrynoderma" and vitamin A deficiency

In temperate countries massive therapy with vitamin A has been used extensively in the treatment of both rare skin diseases, such as Darier's disease, ichthyosis, and pityriasis rubra pilaris, and more common diseases, such as acne vulgaris The extensive literature cannot be quoted here, but has been reviewed by Moore (1957) It seems improbable that any of these diseases are due to lack of vitamin A in the diet Some cases are benefited by dosing with vitamin A, but others are not Cures may be only partial, and sometimes may last only as long as the massive therapy is continued

A report by Fisher (1955) on the treatment of ichthyosis vulgaris with vitamin A may be mentioned both because it provides a good example of the degree of success to be expected, and also because it illustrates the significance of group differences in vitamin A metabolism In children or adults, treated with 50,000–400,000 I U of vitamin A daily, the abnormal scales usually disappeared after 3–40 weeks Even after prolonged dosing, however, some of the patients were not completely cured

Estimations of blood vitamin A before dosing was started gave a low mean value and a lower range of values in the ichthyotic patients than in normal controls, but the ranges for the two groups overlapped Thus the higher values in the ichthyotic group, in spite of the lower average, were much higher than the lowest values in the control group Estimations carried out after dosing again showed a lower mean in the ichthyotic group than in the normal group, but with all values gr[illegible]ased above the

values found before dosing An interesting point was the lack of correlation in individual subjects between a low resting level of vitamin A and ability to benefit from therapy The massive dosing therefore appears to have had some action beyond correcting a low resting level of the vitamin

5 *The Vitamin A Status in Common Diseases*

Lack of space prevents more than brief reference to the wealth of information about the effect of common diseases on the metabolism and storage of vitamin A In many conditions the danger of an induced deficiency of the vitamin must not be overlooked Thus fevers cause a marked lowering of the level of vitamin A in the blood, which is sometimes accompanied by excretion of the vitamin in the urine Chronic nephritis usually involves loss of vitamin A in the urine and the liver reserves of vitamin A usually fall to about one tenth of those found in normal subjects Diseases involving defective absorption of fat such as steatorrhea and celiac disease, inevitably affect the absorption of vitamin A and carotene We have already seen, from the classic work of Bloch (1921) that in infants subsisting on diets low in vitamin A the specific lesions of vitamin A may be precipitated by common systemic diseases such as infections In other infants vitamin A deficiency seemed to be provoked by a simultaneous deficiency of protein, which results in the condition now known as kwashiorkor

For a more adequate review of this field the reader is referred elsewhere (Moore, 1957) A recent paper by Adams and his colleagues (1960) however seems worth special mention These workers detected vitamin A deficiency, indicated in four instances by defective dark adaptation and in one by hyperkeratosis, in patients who had been subjected to total gastrectomy from one and a half to thirteen years previously The levels of vitamin A in the blood of all the patients were very low Dosing with vitamin A increased the levels of vitamin in the blood, and corrected the abnormal dark adaptation It appears therefore that vitamin A should be added to the list of vitamins headed by vitamin B_1 which must be specially provided for gastrectomized patients

V Conclusion

The mass of information which has been under review presents so many diverse facets that a neat and satisfying summary seems an unattainable goal The best that can be done is to connect the various fields by a few concluding remarks

First we may comment again on the very wide variety of lesions which are seen in avitaminosis A which may be so different that the effects of deficiency in different species or even in the same species under different

conditions, may seem to be quite unrelated Thus the rat, with acute xerophthalmia as its most obvious injury may seem quite differently affected to the deficient pig, with paralyzed hind legs To continue on this point we are familiar with bone lesions in deficient dogs and calves, but not in rats or man To some extent, obviously, the lesions sustained by individual animals will depend on how long they survive upon their deficient diet We have seen that sometimes the injuries due to deficiency may even be delayed until long after an adequate diet has been restored

As our next point we may turn to the gaps between the lesions which have been found under experimental conditions and those which have been found to occur spontaneously As far as the author is aware, neither xerophthalmia, nor any other sign of vitamin A deficiency, has ever been known to occur spontaneously in a rat Similarly there is no evidence that dogs suffer spontaneously from bone lesions due to lack of vitamin A It is clear, however, that human subjects can suffer spontaneously from xerophthalmia, and that calves can suffer spontaneously from bone lesions The impression may be formed that any animal could be made to suffer from any of the known effects of avitaminosis A by carefully controlled experimental treatment The nature of the lesions that are sustained spontaneously may depend on the growth pattern of the animal, and on the dietary defects other than lack of vitamin A, to which it is subjected

A most important question, for which a satisfactory answer is still awaited, is the possibility that partial deficiency of vitamin A may cause injury to the animal, without the development of any specific symptoms Does an animal with a large liver reserve of vitamin A, and with the ability to mobilize the vitamin into the blood stream rapidly, possess any advantage over an animal that is less well equipped? In common diseases which are known to affect the vitamin A status is it advisable to resort to dosing with vitamin A? Answers to such questions are urgently required but will undoubtedly be difficult to obtain

A final word may be said on the histological changes that underlie all the diverse injuries seen in vitamin A deficiency As a working hypothesis it may be helpful to regard vitamin A as exercizing a controlling function over the transformation of certain types of cells from the living state into structural units of the body In such cells we may perhaps regard avitaminosis A as causing premature senility and death The thick, keratinized membranes of avitaminosis A may be made up of cells which have been transformed too early into lifeless, and imperfect structural units

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The Role of Vitamin A Acid

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I Introduction

The part that vitamin A plays in vision, though of high importance, is not its principal activity No animal dies of night blindness It is clear that vitamin A must play some very general role in cellular metabolism or cell structure, a role perhaps particularly associated with epithelial cells, since these undergo such marked changes early in vitamin A deficiency

Yet until now the visual role of vitamin A is the only one that has been clearly understood In a recent attempt to pursue this further, in particular to clarify the mechanism of night blindness in vitamin A deficiency, we followed simultaneously a complex of biochemical physiological, and anatomical changes in single groups of rats, held on a vitamin A deficient diet (Dowling and Wald, 1958) It was observed that after the initial stores of vitamin A in the liver and blood had been exhausted the level of rhodopsin in the retina began to fall, the visual threshold reciprocally rising marking the beginning of night blindness Several weeks later the level of

[1] This investigation has been supported in part by grants from the Rockefeller Foundation the Office of Naval Research and the United States Public Health Service The material of this paper has been reported earlier (Dowling and Wald 1960) Part of this work was done while J E D held a Post Sophomore Fellowship of the Public Health Service while on leave from the Harvard Medical School (1959–1960)

We should like to thank Dr Otto Isler of Hoffmann La Roche Basle Switzerland and the Organic Research Laboratory of Distillation Products Industries Rochester New York for generous gifts of vitamin A acid

opsin in the retina also began to decline, and at this time the r
segments deteriorated anatomically

One reason to have begun these experiments was to test a hy
that had been suggested earlier for this second type of effect (Walc
We had already learned that opsin in solution is a much less stable
than rhodopsin It is readily denatured by exposure to heat (H
1958–1959), acids, and alkalis (Radding and Wald, 1955–1956), ur
cumstances that leave rhodopsin intact When as the result of the c
diet, the retina begins to contain opsin that can find no vitamin
which to combine, it seemed reasonable to suppose that such opsi
disintegrate, and since the rod outer segments are largely composec
protein, that their structure must deteriorate accordingly

The difficulty is that just at the time when the rod outer segme
teriorate in a vitamin A deficient rat, all the classic overt signs of
A deficiency appear loss of weight, postural imbalance, respirato
turbances, corneal opacities, disarrangement of coat, and red se
about the eyes It is clear that tissues have begun to disintegrate i
parts of the body, and within a few more days all such animals ar
or dying That is, having attempted to identify and clarify a spe
quence of events in the outer segments of the retinal rods, we obta
only is one detail in a complex of changes, spread widely through
animal

It was at this point that we turned to a study of vitamin A aci
substance, $C_{19}H_{27}COOH$, was prepared by Van Dorp and Arens (
and shown by them to possess high vitamin A activity in the rat (
Indeed, when the acid is given orally as the sodium salt, it is said
as active in supporting growth as vitamin A alcohol fed orally Eve
massive doses of vitamin A acid were injected or fed orally, howev
storage of vitamin A could be detected in the liver (Arens and Van
1946) Nor could the acid itself be found in the body tissues, eve
massive dosage with this substance (Sharman, 1949) The action o
min A acid in the rat, therefore, does not seem to involve its reduct
vitamin A There is no evidence that the rat is able to perform this
tion Rather, it seems that the acid itself, or some further derivati
tained from it, supports the growth of the animal

Several years ago Moore (1953, 1957), discussing these experi
suggested that though vitamin A acid can fulfill some of the general
tions of vitamin A in the tissues, its inability to be reduced to the al
and hence also to the aldehyde, retinene, might make it unavailab
vision That is, Moore suggested that vitamin A acid, while supp
the growth of rats, might not prevent the development of night blin

This has now proved to be the case Young rats, placed on vitamin

ficient diets supplemented with vitamin A acid, grow very well and continue to look altogether sound Eventually, however, they become highly night blind, more so than we have ever observed in animals on a simple vitamin A deficient diet The animals maintained on vitamin A acid, therefore, present us with an isolated sequence of visual and retinal changes, uncomplicated by general tissue deterioration, and also clarify to a degree the role of vitamin A acid, and the interrelationships between vitamin A and its acid in general metabolism

II Methods

Male, weanling rats from the Harvard colony were raised on the standard U S P vitamin A deficient test diet to which supplements were added as wanted The techniques for evaluating liver and blood vitamin A, rhodopsin, and opsin, and recording electroretinograms, have been described earlier (Dowling and Wald 1958)

Vitamin A and vitamin A acid dissolved in cottonseed oil, were administered by mouth, through a syringe with a blunted point Since in these experiments we were interested only in maximal effects of vitamin A acid, excessive doses were given Three feedings a week provided a dosage level equivalent to at least 50 μg per day chosen with the thought that if the acid possesses the lowest activity yet reported for it—10% as high as vitamin A (Van Dorp and Arens 1946b)—we should still be providing about twice the vitamin A activity considered to be adequate for the rat (2–2 5 μg vitamin A per day)

III A Typical Experiment

The general nature of the observations is best illustrated with such a typical experiment as that shown in Fig 1 Two littermates were placed on the deficient diet supplemented in one of them with vitamin A acid Both animals grew at about the same rate for 5–6 weeks Then the unsupplemented animal stopped growing rapidly lost weight, and died on the 57th day of the diet The other animal continued to grow and appeared to remain in fine condition as the photograph taken on the 157th day of the diet is meant to show On the same day this rat's electroretinograms (ERG) were recorded, as shown at the right in Fig 1 compared with those of a normal animal measured at the same time

This animal, though normal in weight and appearance, was highly night blind Its visual threshold—the luminance of a 1/50 second flash needed to excite a just measurable ERG—was 3 25 log units (about 1800 times) above normal As the figure shows this animal yielded about the same ERG at log luminance 4 as the normal animal did at log luminance 0 Only 1–5% of normal amounts of rhodopsin could be extracted from the

retinas of animals in this condition. This represented a higher degree of night blindness than we had ever observed in rats kept on the vitamin A deficient diet without supplementation.

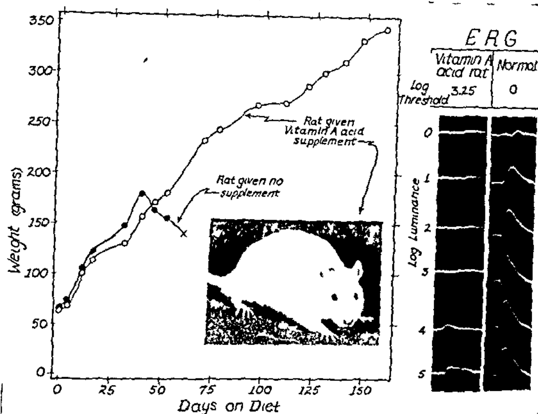


FIG. 1. Nutritional activity of vitamin A acid. Littermates had been placed on a vitamin A deficient diet, supplemented in one of them with vitamin A acid. The rat given no supplement died on the 57th day of the diet; the animal receiving vitamin A acid continued to grow and remained in excellent condition for the duration of the experiment, a little over 5 months. The picture of this animal was taken at the end of the experiment, as were the electroretinograms shown at the right, compared with those of a normal animal. They show this rat to be highly night blind: its visual threshold had risen 3.25 log units (about 1800 times) above normal, and only just detectable ERG's could be evoked at even the highest luminances.

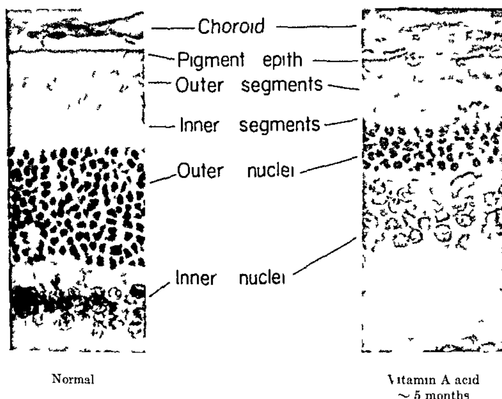
Next day these animals were sacrificed, and the retinal histology was examined (Fig. 2). In the animal kept for 5 months on vitamin A acid, all the retinal tissues appeared normal, except for the visual cells. The pigment epithelium, bipolar layer, and ganglion cell layer did not seem changed in any way.

The nuclei of the visual cells (outer nuclear layer) were considerably reduced in number, but otherwise appeared normal. The reduction in the

number of visual cells probably accounted also for a thinning out and compression of the layer of inner segments

In this retina, however the rats no longer possessed outer segments With the loss of rhodopsin, these organelles which are largely composed of rhodopsin, had almost vanished

In such an animal we can follow as an isolated condition, uncomplicated



Normal

Vitamin A acid ~ 5 months

FIG 2 Retinal histology of the night blind animal shown in Fig 1 compared with that *of a normal animal All the retinal tissues are normal except the visual* cells which are reduced in number and almost completely lack outer segments

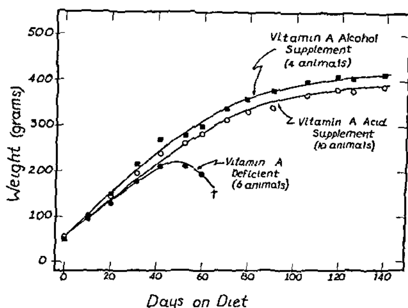
by general systemic deterioration the changes that characterize the development of dietary night blindness On removal of vitamin A and with it depletion in the liver and blood the rhodopsin concentration falls and the visual threshold rises (night blindness) Then perhaps in part because deprived of the stabilizing effect of its prosthetic group opsin decays, with the consequent anatomical deterioration of the rod outer segments

In *this way* the supplementation with vitamin A acid results in an animal which appears physiologically normal except for its night blindness, biochemically *normal except for its lack of rhodopsin and opsin* and anatomically normal except for the almost total loss of the outer segments

rods By the same token, this animal seems to indicate that the only ion in the body that requires vitamin A as the alcohol may be the ation of visual pigments All the general systemic functions of vitamin pear to be performed by vitamin A acid These possibilities are ex ed in what follows

IV Growth and Maintenance

anling rats were divided into three groups, all placed simultaneously e standard vitamin A deficient test diet One group was supplemented



3 Growth of animals on a vitamin A deficient diet compared with growth same diet supplemented with 50 μg per day of vitamin A or vitamin A acid nsupplemented animals lost weight after 6 weeks on the diet, and all had died end of the eighth week The supplemented animals grew as well on vitamin A on vitamin A

vitamin A, the second with vitamin A acid, both dissolved in cotton oil, and the third group was given the same amount of cottonseed one

growth of these animals is shown in Fig 3 For the first 5 weeks on et, all three groups grew about equally Then the unsupplemented ls stopped growing declined rapidly in weight and all had died by nd of the eighth week The animals receiving supplements of vitamin l of vitamin A acid grew regularly throughout the experiment (140 The small difference in average weight shown in Fig 3 (27 gm , does not appear to be significant Each of the supplemented groups l considerably in weight, and they overlapped each other widely min A 360–450 gm , vitamin A acid 318–512 gm) In another ex

periment, after 135 days of a similar regime the average weight of the animals on vitamin A acid was slightly *greater* than that of the animals receiving vitamin A (324 314 gm) It may be concluded that the growth of the animals on vitamin A acid was entirely normal

Both groups of animals also appeared equally sound, externally and in the gross appearance of the internal organs on autopsy The tracheal epithelium, examined under the microscope, also appeared normal in both groups

To test the biological effectiveness of vitamin A acid further, this supplement was withheld in other experiments until the fourth to seventh week of the vitamin A deficient diet, that is, until animals had stopped gaining or were rapidly losing weight On administration of vitamin A acid such animals immediately began to grow and soon appeared identical with those supplemented from the start of the experiment Also any signs of vitamin A deficiency that had developed on the deficient diet rapidly healed except for occasional scars on the cornea owing to earlier xerophthalmia which remained permanently

V Storage and Depletion of Vitamin A

We had noticed that night blindness develops equally rapidly in rats on a vitamin A deficient diet whether or not supplemented with vitamin A acid During the first weeks on the diet such animals use up the supplies of vitamin A stored in the liver and it seemed from this observation that the rate of depletion of vitamin A in the liver is probably equally rapid, whether or not vitamin A acid is available

Figure 4 shows this to be the case The vitamin A content of the liver was measured in animals divided into the three dietary groups already described, indeed these animals formed part of the same experiment shown in Fig 3 Those receiving supplements of vitamin A (50 μg per day) rapidly increased their stores in the liver Those supplemented with vitamin A acid however lost their initial stores of liver vitamin A as rapidly as the rats receiving no supplements Both groups also developed night blindness at the same time Vitamin A acid not only fails to contribute vitamin A to the liver, but seems to have no sparing action on the vitamin A already there

Since the animals receiving the acid supplement are adequately maintained by it, except for their vision the liver seems to lose its vitamin A independently of demand We shall have more to say of this relationship below These observations also raise the question, in an animal optimally supplied with vitamin A acid how much vitamin A would be needed to maintain normal vision? Would it be less than, or the same as, the amounts in animals not receiving vitamin A acid? We have not yet made such determinations

The animals supplemented with vitamin A acid fail to store this substance Vitamin A acid is readily identified by the sharp absorption band at 573 mμ which it yields in the antimony chloride test We have never by this means been able to detect the acid in extracts of the liver, kidney, or blood of animals receiving large amounts of this substance in the diet (cf also Sharman, 1949)

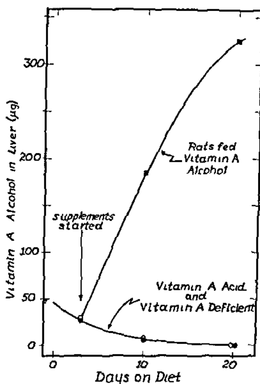


FIG 4 Total vitamin A content of the liver in animals placed on a vitamin A deficient diet and on the same diet supplemented with 50 μg per day of vitamin A or vitamin A acid (These animals formed part of the same experiment as in Fig 3) The animals supplemented with vitamin A rapidly increased their liver stores Those receiving vitamin A acid lost their initial stores of vitamin A as rapidly as those receiving no supplementation

One consequence of the failure to store either vitamin A or the acid is that these animals though normal in weight and in excellent health, fail very quickly on interruption of the supplementation (Fig 5) Animals growing well on vitamin A acid, stop growing within a few days when the supplement is removed, decline rapidly in weight and within 3–5 weeks die displaying all signs of severe vitamin A deficiency That is, these animals respond like young rats on a vitamin A deficient diet after all internal stores of vitamin A have been exhausted If the supplementation with vitamin A acid is renewed, even in terminal stages of the deficiency such animals rapidly recover (Fig 5)

whether supplemented with vitamin A acid from the start, or from the fifth week—when they had stopped growing—or from the seventh week—when the weight was declining rapidly—continued to grow and to become more night blind

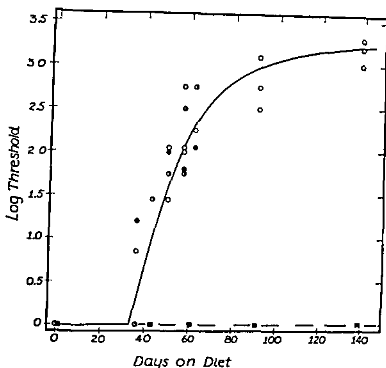


FIG 6 Visual thresholds of animals kept on a vitamin A deficient diet (●), and on the same diet supplemented with vitamin A (■) or vitamin A acid (○) (same experiment as in Fig 3) The threshold is the smallest luminance of a $\frac{1}{50}$ second flash of light needed to evoke a just perceptible ERG The vitamin A supplemented rats formed the control group whose thresholds are arbitrarily given the value 1 (log threshold = 0) All other thresholds are expressed relative to these and represent therefore increments of log threshold above the control level In the animals supplemented with vitamin A acid the threshold rises as soon and as rapidly as in those receiving no supplementation All the latter group have died, however by the end of the ninth week, whereas the vitamin A acid animals survive to grow more night blind The thresholds level off after 12–15 weeks on the diet at about 3 25 log units (about 1800 times) above normal At this time the retinas contain only 1–5% of the normal amounts of rhodopsin

In both these experiments the visual thresholds do not increase indefinitely, but level off after 12–15 weeks at 3 3–3 5 log units—about 2000–3000 times—above normal The reason for this is still not clear Direct extraction of the retinas of such animals showed that they contain about 1–5% of the normal amounts of rhodopsin

The electroretinograms of animals maintained on vitamin A acid are shown in Fig 8 The stimulus was a $\frac{1}{50}$ second flash of light, the lumi

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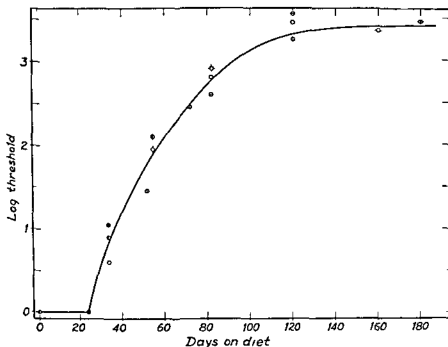


FIG 7 Development of night blindness in animals on a vitamin A deficient diet (●) and on the same diet supplemented with vitamin A acid from the start (○) or from the fifth week (⦶)—when the animals had stopped growing—or from the eventh week (⊖) when they were declining rapidly in weight In all these animals the visual threshold begins to rise at the same time and rises equally rapidly By the end of the eighth week the unsupplemented animals have died whereas those receiving vitamin A acid grow increasingly night blind After about 18 weeks on the diet the visual threshold of the latter group becomes constant at about 3 3 log units (about 2000 times) above the normal level

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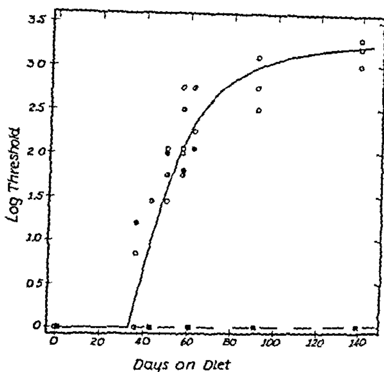


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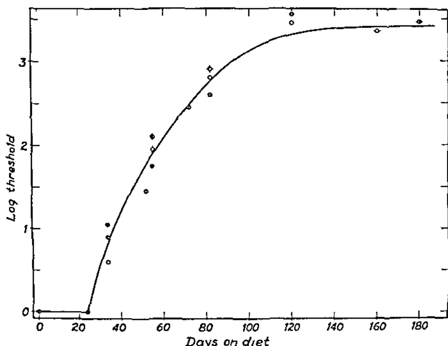


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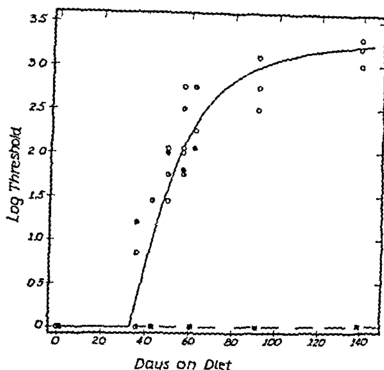


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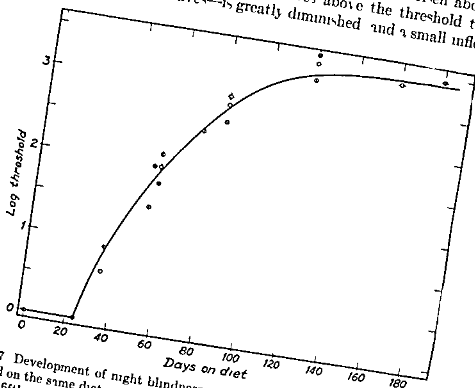


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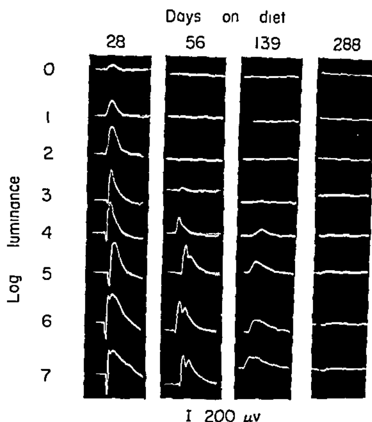


FIG. 8. Electroretinograms of animals on a vitamin A deficient diet supplemented with vitamin A acid. Responses to a $\frac{1}{500}$ second flash of light at luminances ranging over 7 log units (1–10 million). The initial deflection downward is the negative a wave; the later sweep upward is the positive b wave. The first strip of records was made on the 28th day of the diet, when the ERG is still normal. On the 56th day the animal is typically night blind; its threshold has risen about 500 times; the a and b waves have decreased greatly in amplitude; and a small inflection on the downward sweep of the b wave has separated off as a second positive wave. By the 139th day, the threshold has risen only moderately further; but now increasing the brightness of stimulus above the threshold level has little effect. The retina is losing the capacity to generate an ERG. By the 288th day (10 months on the diet) no response can be elicited at the highest available brightness. The animal is now blind.

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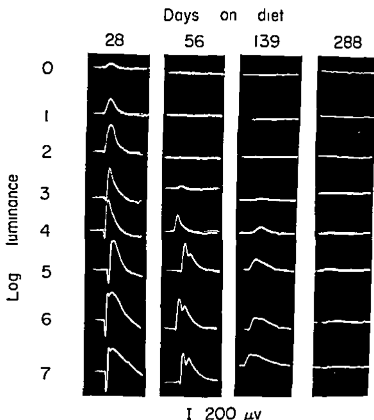


FIG. 8. Electroretinograms of animals on a vitamin A deficient diet supplemented with vitamin A acid. Responses to a $^{1}/_{30}$ second flash of light at luminances ranging over 7 log units (1: 10 million). The initial deflection downward is the negative a wave, the later sweep upward is the positive b wave. The first strip of records was made on the 28th day of the diet, when the ERG is still normal. On the 56th day the animal is typically night blind; its threshold has risen about 500 times, the a- and b waves have decreased greatly in amplitude, and a small inflection on the downward sweep of the b wave has separated off as a second positive wave. By the 139th day, the threshold has risen only moderately further, but now increasing the brightness of stimulus above the threshold level has little effect. The retina is losing the capacity to generate an ERG. By the 288th day (10 months on the diet), no response can be elicited at the highest available brightness. The animal is now blind.

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of rhodopsin extracted from the retina We had found earlier that in vitamin A deficient animals receiving no supplementation the logarithm of the threshold rises linearly as the rhodopsin content falls (Dowling and Wald 1958) In animals supplemented with vitamin A acid this relationship can be extended further As Fig 9 shows it is maintained over the whole range of the measurements

Figure 9 involves a further comparison The rhodopsin content of the retina falls in vitamin A deficiency it falls also on exposure of the animals

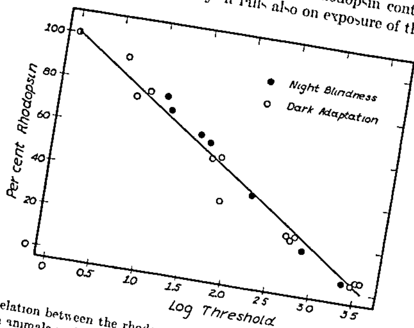


FIG 9 Relation between the rhodopsin content of the rat retina and the visual threshold in animals night blind owing to vitamin A deficiency and in normal animals dark adapting after exposure to bright light In both instances the same relationship is observed the log threshold rises linearly with fall in rhodopsin content that is the log sensitivity (log 1/threshold or −log threshold) varies linearly with the rhodopsin concentration

to bright light (light adaptation) rising again when the light is extinguished That is vitamin A deficiency and light adaptation are two ways of inducing night blindness How does the relationship between rhodopsin content and log threshold compare in both conditions? To answer this question a group of normal rats was highly light adapted then the light was shut off and periodically an animal was anesthetized and its ERG threshold determined The same and also other groups of animals were light adapted similarly and after the same intervals in darkness animals were killed and their retinas extracted for rhodopsin In this way the relationship between visual threshold and the retinal content of rhodopsin was measured this time

however in normal animals in the ordinary course of dark adaptation (Dowling, 1960) [2]

Figure 9 shows that a change of rhodopsin concentration in the retina, whether induced by visual adaptation or by vitamin A deficiency has the same effect on the threshold In both conditions the logarithm of the threshold rises linearly as the rhodopsin content falls, a relationship described by the equation, $\log (I_t/I_0) = 3.6\,(R_0 - R_t)/R_0$, in which I_0 and R_0 are, respectively, the threshold and rhodopsin concentration in dark adapted control animals, and I_t and R_t are respectively the thresholds and rhodopsin concentrations in vitamin A deficient or incompletely dark adapted animals The identity of the effects of vitamin A deficiency and visual adaptation implies that up to this point dietary night blindness is accounted for completely by the loss of rhodopsin from the retina

VII Anatomical Changes

We have already seen that by the twenty fourth week on vitamin A acid, the visual cells are considerably reduced in number and most of the rods lack outer segments (Fig 2) The disintegration of the rod outer segments seems to accompany the loss of opsin For example, in one experiment, in the eighteenth week of the deficiency (with vitamin A acid supplementation), when the threshold had risen over 3 log units, and the rhodopsin level was only a few per cent of normal the opsin had declined to half the normal level

As the retina loses opsin, examination in the electron microscope shows progressive deterioration of the microstructure of the rod outer segments The electron microscopy was done in collaboration with Dr I R Gibbons, and will be reported in detail elsewhere (Dowling and Gibbons, 1960) After removal of the cornea and lens, the whole fundus of the eye was fixed for 1 hour in buffered osmium tetroxide, washed, dehydrated with acetone, and embedded in Araldite resin Ultrathin sections were cut on a Porter Blum microtome and examined in the R C A EMU 3D electron microscope

Figure 10 shows a longitudinal section through portions of the rod outer segments of a normal rat As in other animals, the outer segment consists of a stack of flattened, hollow disks, enclosed within an outer membrane The outer segment is about 1 5 μ wide and about 15 μ long It contains about 35 disks per micron or about 525 disks in all

[2] The first such measurements were performed in normal and vitamin A deficient rats by Tansley (1931) She assumed as the simplest relation that the sensitivity (1/threshold) is proportional to the concentration of r … e find the logarithm of the sensitivity to be lin ar with rhodopsin concer … pare also ophthal mo copic measureme … eaching and reger … dopsin in the rat retina during light a … tion by Lewis (

Figure 11 shows an early stage in the deterioration of this structure typical of animals kept 3–4 months on the vitamin A deficient diet supplemented with vitamin A acid Many of the retinal disks are still intact but many others have segmented, or are in process of doing so into groups of distended vesicles and tubules

By 6 months on the regime these changes have progressed further (Fig

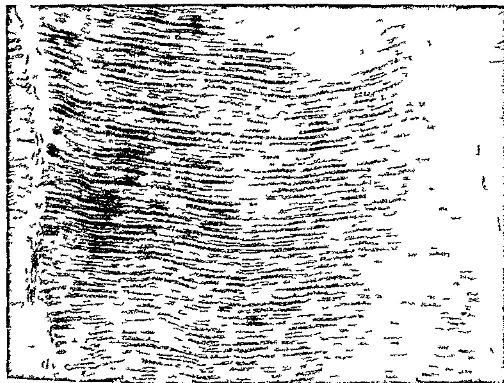


FIG 10 Electron micrograph of a longitudinal section through a rod outer segment and portions of two neighboring outer segments in the normal rat As in other animals the outer segment consists of a stack of transverse flattened hollow disks all enclosed in an outer membrane The rat outer segment is about 1 5 μ wide and 15 μ long and contains about 525 disks Magnification ×58 000

12) Few outer segments remain and their internal structure is highly disorganized They have lost also their characteristic cylindrical shape becoming distorted and tending to collapse often into roughly spherical shapes By this time also the visual cells are considerably reduced in number and their inner segments formerly long and slender have shortened and broadened (Fig 2) The ultrastructure of the nuclei and inner segments of the visual cells however, has not visibly changed

As animals continue longer on this regime the visual cells undergo further alterations Figure 13 shows sections of the retinas of two litter

mates, one supplemented with vitamin A, the other with vitamin A acid for 10 months on the otherwise A deficient diet. The animal receiving vitamin A acid no longer yielded an ERG in response to the brightest lights available in our apparatus (Fig. 8). In this retina, the pigment epithelium and the layers of bipolar and ganglion cells still look normal. The visual



FIG. 11. Electron micrograph of a longitudinal section through the rod outer segment in a highly night blind rat, typical of animals that had been 3-4 months on a vitamin A deficient diet supplemented with vitamin A acid. Some of the transverse disks appear intact; others have segmented into groups of distended vesicles and tubules. Magnification ×90,000.

cells, however, are reduced to a single row of nuclei, and the individual cells have contracted to a cuboidal shape, almost wholly occupied by the nucleus, with no distinguishable inner or outer segment. In this extraordinary state the pigment epithelium lies in direct contact with the layer of visual cell nuclei.

VIII. RECOVERY

On administration of vitamin A, animals which had become night blind on the vitamin A deficient diet supplemented with vitamin A acid recover

in varying degree When such animals have been on the diet up to 10 weeks recovery ordinarily is rapid and complete Within several hours after feeding a large dose of vitamin A in cottonseed oil the visual threshold—which



FIG 12 Electron micrograph of the longitudinal section through a rod outer segment in an animal that had been 6 months on the vitamin A deficient diet supplemented with vitamin A acid Few rod outer segments remain in such a retina and as in this example they are highly distorted in shape and micro tructure A few isolated groups of disks still appear but mainly the internal structure has degenerated into distended tubules and vesicles The outer segments have also lost their normal cylindrical shape, and collapsed to irregular ellipsoids or spheres Magnification ×61 000

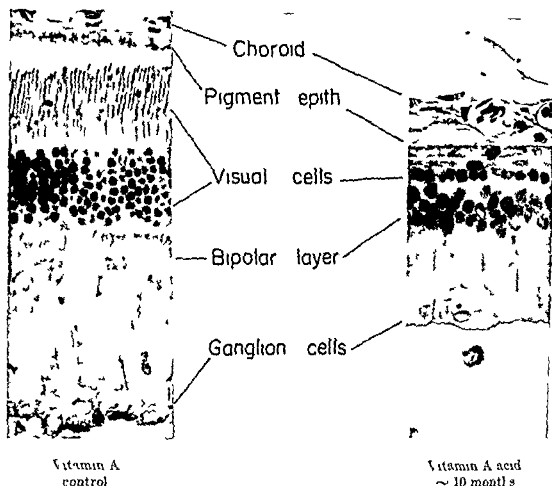
may by then have risen about 2 log units above normal —begins to fall and within 2–3 days has reached the normal level

After longer periods on the diet, recovery is more complex Figure 14 shows the effect of one intraperitoneal injection of 1 mg of vitamin A on two animals that had been on the diet for 25 weeks These animals recover in two stages The visual threshold first falls rapidly for 40–50 hours reaching a level about 1 log unit above normal Thereafter it continues to fall slowly over a period of several weeks of further vitamin A supplementation

In animals kept still longer on the diet, these two phases of recovery tend

to reverse their proportions, the fast phase contributing less and the slow phase accounting for more and more of the total change

It seems probable that these two stages of recovery are concerned with



Vitamin A control

Vitamin A acid ~ 10 months

FIG 13 Sections through the retinas of two animals—littermates—both of which had been 10 months on the vitamin A deficient diet supplemented with vitamin A (left) or with vitamin A acid (right) The retina on the left is normal that on the right is also normal except for its visual cells The visual cells only about one tenth of which remain form a single almost complete row of nuclei directly apposed to the pigment epithelium No inner or outer segments are visible Such an animal is not only night blind but blind (cf Fig 8 ERG s at 288 days) and does not regain its vision after months of vitamin A supplementation

reversing the two phases we have described in the development of night blindness first the rise of visual threshold associated with the decline of rhodopsin concentration owing to simple lack of vitamin A, later the loss of opsin and anatomical deterioration of the rods It seems reasonable to suppose that the fast phase of recovery involves the combination of any opsin that remains with the vitamin A that is administered, whereas the

synthesis of opsin and structural repair of the visual cells occupy the slow phase Certain instances in which the recovery from experimental human night blindness has exhibited similar fast and slow phases may perhaps be explained in the same way (Hecht and Mandelbaum 1938 1939 1940 McDonald and Adler, 1940)

In animals kept longer than 6 months on the diet the visual threshold

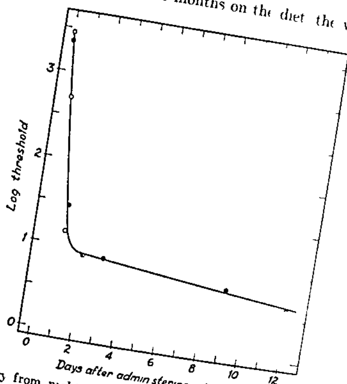
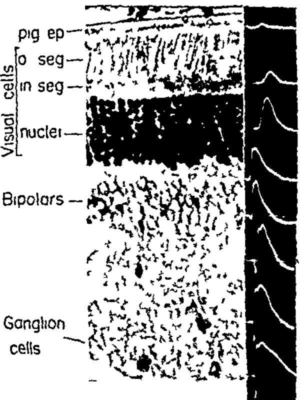
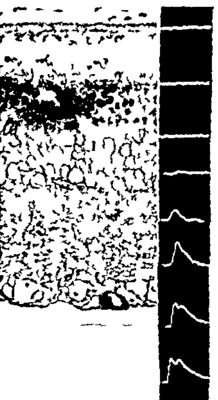


FIG 14 Recovery from night blindness on administration of vitamin A Five animals that had been kept for 25 weeks on the vitamin A deficient diet supplemented with vitamin A acid received an intraperitoneal injection of 1 mg vitamin A in cottonseed oil followed by regular dietary supplementation with vitamin A The visual threshold starting from levels 3 3–3 4 log units (about 2000 times) above normal first falls rapidly to a level about 1 log unit above normal Thereafter it continues to fall more slowly for several weeks returning almost to the normal level (cf Fig 15)

no longer recovers completely even after months of vitamin A supplementation This irreversible aspect of night blindness is probably associated with the loss of visual cells which as is true of nerve cells generally in adult mammals once lost are never regained

A decline in the density of visual cells must raise the visual threshold just as a decrease in the area of the visual field normally raises the threshold and so should be a source of various degrees of permanent night blindness If indeed it is true that a decrease in the density of visual cells has about the same effect as a decrease in the area of the visual field it should raise


Vitamin A control
pig ep
o seg
in seg
nuclei
Visual cells
Bipolars
Ganglion cells


Vitamin A acid

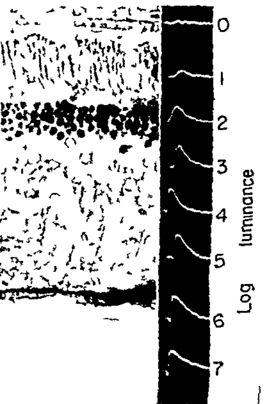

~ 6 1/2 months
+ 16 days vitamin A
0
1
2
3
4
5
6
7
Log luminance

Fig 15

the threshold by a factor corresponding to the reciprocal of this decrease (Ricco's law) or to the square root of the reciprocal (Piper s law) That is, in such a condition as shown in Fig 2, in which the number of visual cell nuclei has been reduced to about $\frac{4}{9}$ if as a result of vitamin A administration all the remaining cells again become fully functional, without increasing in number, the final threshold should be raised permanently by $^{9}/_{4}$ or by $\sqrt{9/4}$ (i e , 2 25 or 1 50 times) over the normal threshold Such changes do not seem large on a logarithmic scale they correspond to raising the threshold 0 35 or 0 18 log unit

A typical example of such an experiment is shown in Fig 15 Three littermates were kept on the vitamin A deficient diet for 6½ months one supplemented with vitamin A to serve as control the other two supplemented with vitamin A acid Then the ERG's of the control and one vitamin A acid animal were measured and on the following day the control and the other vitamin A acid animal were killed and their retinas sectioned

The retina and ERG's of the control animal were normal (Fig 15, left) The vitamin A acid animals however displayed the usual anatomical and physiological signs (Fig 15 center) In the retina only remnants of outer segments remained and the inner segments were few and stumpy in appearance The layer of visual cell nuclei was reduced from the normal 9 to about 4 rows The ERG record shows the mate of this animal to have been highly night blind the visual threshold had risen more than 3 log units and the ERG s at higher luminances displayed all the usual changes that go with this condition

The surviving vitamin A acid animal was supplemented for 16 days with vitamin A Then his ERG's were remeasured and the retinas were sectioned immediately afterward (Fig 15 right) In the retina the visual cells seem to have been completely repaired The outer segments are long and well

Fig 15 Development of and recovery from night blindness Three littermates had been kept for 6½ months on a vitamin A deficient diet supplemented in one with vitamin A to serve as control and in the others with vitamin A acid The ERG responses and retinal histology of the control animal are shown at the left At this time the ERG s of one of the vitamin A acid animals were also measured the other one was killed and its retinas sectioned (center) The ERG s show a high degree of night blindness and the retina the almost complete loss of outer segments and reduction of the number of visual cells to about $^{4}/_{9}$ the normal population (compare Figs 1 and 2) The surviving vitamin A acid animal was now given vitamin A supplementation for 16 days Then its ERG s were remeasured and its retinas sectioned immediately afterward (right) The rods are still reduced to about $^{4}/_{9}$ the normal number but seem to have recovered their normal structures The visual threshold has returned to about 0 25 log unit above the normal level but the ERG s at higher luminances are much reduced in amplitude compared with the control This small elevation of visual threshold above normal and decrease in the size of the ERG are apparently permanent effects owing to the reduction in number and density of visual cells

developed The inner segments are shorter and broader than in a control retina, but otherwise normal in appearance The visual cells, however, had not increased in number, and only about ⅙ the normal population remained

Under these circumstances the electroretinograms display an interesting condition As we had expected, the visual threshold, though again nearly normal remained about 0 25 log unit above that of the control littermate, owing apparently to the decrease in number of visual cells At higher levels of stimulation, the ERG's displayed a further effect though once again normal in shape, they were considerably reduced in amplitude, presumably because generated in such an animal by a greatly reduced density of visual cells Probably one could mimic all these effects in a normal animal by reducing the area of the visual field, and hence the numbers of visual cells affected by the stimulus [3]

An animal kept as long as 10 months on the diet, in which, as shown in Fig 8 even our brightest stimuli failed to evoke an ERG, displays no perceptible recovery even after months of high supplementation with vitamin A Such an animal is probably permanently blind It still possesses visual cells, though much reduced in number—perhaps still such a row of visual cell nuclei as in Fig 13 It fails to recover measurable visual responses apparently because such visual cells as shown in Fig 13 can no longer regenerate outer segments The outer segments are derived embryonically from potential cilia or flagella, springing in the cell from a basal body and "root" (de Robertis, 1956) In such visual cells as in Fig 13, the latter structures have been lost, and with them apparently the capacity to form a new outer segment

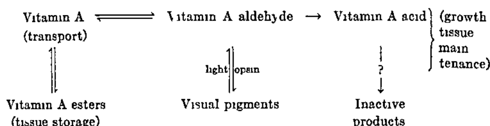
IX Discussion and Conclusions

It appears from these experiments that the only function vitamin A may perform directly in the rat is to supply the prosthetic group of its visual pigments All other functions—growth, general tissue maintenance—are served equally well by vitamin A acid

The rat, however, seems unable to reduce the acid to the aldehyde or alcohol as shown by the observation that no matter how high the level at which the acid is fed, no vitamin A is deposited in the liver, nor is any visual pigment synthesized For this reason animals kept on a vitamin A deficient diet supplemented with the acid, though they grow normally and remain in excellent health become night blind, indeed more night blind than they can survive to become on a vitamin A deficient diet alone

[3] It is of interest in this connection that Brindley (1956) has found the size of the ERG in frogs to be roughly proportional to the area of retina illuminated

The general metabolism of vitamin A seems therefore to involve the following relationships (compare Moore, 1957)



Vitamin A ordinarily is stored in the tissues, principally in the liver, as esters It is transported in the blood mainly as the free alcohol (Glover *et al*, 1947, Krinsky *et al* 1958) The equilibrium of the alcohol dehydrogenase system which oxidizes it to retinene lies far over toward reduction (Bliss, 1951), no appreciable oxidation to retinene occurs unless the latter is re moved as fast as formed In the retina this is accomplished by opsin trap ping the retinene to form the visual pigments (Wald and Hubbard, 1950) [4] The irreversible removal of retinene by oxidation to vitamin A acid may provide a second such mechanism

The general growth and tissue functions of vitamin A appear to be ful filled either by vitamin A acid itself (presumably in a combined or activated form) or by some product of its further metabolism Experiments are in progress to determine what the active principle may be

It is already clear, however, that neither vitamin A acid itself, nor any derivative of it that possesses biological activity, is stored by the animal Shortly after feeding the acid, none of it can be found in the blood liver, or kidney, and if active derivatives were stored we should find that animals which had been plentifully supplied with vitamin A acid over long periods could tolerate removal of this supplementation longer than they do Our experiments show that such animals stop growing within a few days of deprivation and develop severe symptoms of vitamin A deficiency within 1–2 weeks (cf Fig 5)

That is, vitamin A acid however effective immediately, does not "buffer" the animal against deprivation Having been formed irreversibly, it is apparently rapidly degraded and it or its derivatives can exercise therefore only a transient function [5] Ordinarily the rat must rely for vitamin A acid

[4] The enzyme retinene reductase mentioned in this paper appears to be identical with liver alcohol dehydrogenase (Bliss 1949)

[5] The degradation of vitamin A in the rat has recently been examined (Wolf *et al*, 1957) Large amounts of vitamin A (3–4 mg) labeled with penultimate C^{14} were injected intraperitoneally Surprisingly large fractions were excreted in the form of water soluble apparently aliphatic compounds in the urine

on the continuous oxidation of its stores of vitamin A It is as the storable precursor of vitamin A acid that vitamin A probably fulfills its most important function

This relationship may explain our observation that vitamin A is depleted from the liver at the same rate, whether or not the rat is supplied with vitamin A acid, as though this depletion were independent of demand by the tissues Such a condition could result automatically from the irreversible oxidation of vitamin A to the acid A given amount—in young rats 2-2 5 μg per day—may be oxidized to the acid in the liver or elsewhere, whether needed or not Alternatively, it is possible that the rat uses vitamin A preferentially as long as it remains available, perhaps because better equipped to transport it among the tissues, or for some other reason

The *animals maintained on vitamin A acid exhibit a sequence of changes* that seem to remain wholly restricted to the visual cells for as long as our experiments have continued first, the decline in rhodopsin concentration, accounting completely for the rise of visual threshold, then the loss of opsin, and attendant disintegration of the outer segments of the rods, by this time also the retraction of the inner segments and irreversible decrease in number of the visual cells Finally only a single, incomplete row of visual cell nuclei remains, devoid of inner segments The animal is now blind, and apparently cannot recover its vision

The retina in this final state (cf Fig 13) resembles closely that described in so called "rodless" mice, a genetic condition associated probably with several distinct recessive mutations (Keeler, 1926, 1937) In one such mutant strain (C3H), the retina develops normally until the mouse is 10 days old, outer and inner segments of the visual cells by then being clearly distinguishable Thereafter, visual cells begin to die, and by the age of about 25 days the retina has reached a state almost identical with that shown in Fig 13 A single row of stripped visual cell nuclei remains, the retina and pigment epithelium appearing otherwise normal, as they continue to do in the adult (Noell, 1958a b)

The same histological pattern appears also in Johnson's description of a rat which, having been severely vitamin A deficient, was allowed to "recover" through 12 weeks of vitamin A supplementation (cf Johnson, 1943 Fig 2D) Again a single row of visual cell nuclei remains, lacking inner or outer segments, in an otherwise relatively normal retina

This condition also is characteristic of the human disease *retinitis pigmentosa* Here again, though not always, the visual cells may be reduced finally to a single row, lacking inner and outer segments, though the bipolar and ganglion cell layers appear to remain intact It has been suggested that this disease may involve some form of localized vitamin A deficiency (Cogan, 1950) One difficulty with this notion is that in true vitamin A defi

ciency, degenerative changes are not confined to the visual cells, but come to involve eventually all the retinal layers (Tansley, 1933, Johnson, 1939, 1943) It is possible, however, as the present experiments show, to induce retinal changes much like those of retinitis pigmentosa by keeping animals on a vitamin A deficient diet supplemented with vitamin A acid The invasion of the retina by pigment is a secondary aspect of this disease and cannot be looked for of course in our albino animals

A characteristic feature of human retinitis pigmentosa is that the rods deteriorate before the cones (Cogan, 1950, Zeavin and Wald 1956) We have some reason to anticipate the same sequence in vitamin A deficiency whether or not supplemented with vitamin A acid Cone visual pigments are synthesized very much more rapidly than rod pigments (Wald *et al*, 1954–1955), so that when vitamin A is in short supply, the cones may capture all they require while the rods go hungry We have not yet succeeded in testing this point adequately a difficult business in any case in the rat, in which cone vision seems to play only a minor role

We have suggested mechanisms for the rise of visual threshold and the loss of rod outer segments in animals kept on the vitamin A deficient diet supplemented with vitamin A acid Why, however, when their other tissues remain intact, do such animals lose entire visual cells? This process seems to bear some relationship to certain types of traumatic degeneration known to occur in the retina and brain [6] So for example, when mammalian optic nerve fibers are cut, not only do their cells of origin, the retinal ganglion cells degenerate ("retrograde degeneration'), but so also do cells of the lateral geniculate nucleus with which they make synaptic connection ("transsynaptic degeneration") Injury commonly has the effect of causing the degeneration of cells in the central nervous system and it has been suggested that the subsequent degeneration of other cells with which they communicate may be caused by *lack of excitation* Either explanation might be applied to the visual cells They may go because the disintegration of their outer segments constitutes an injury,' even though this does not seem to deprive them of any vital part On the other hand, the loss of their outer segments does deprive these cells of stimulation, and this may be a more powerful factor in their decay

The loss of visual cells, so far as our experiments have gone, ends with the tissue reduced to a single layer in which the cells have lost all the special structures that marked them formerly as rods and cones, a condition shared, as already noted with genetically "rodless' mice and certain instances of human retinitis pigmentosa At this point what remains of the layer of visual cells has taken on the appearance of a simple, cuboidal

[6] Compare Polyak s discussion (1941) of axonal (retrograde) and transneuronal degeneration

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Summary Discussion

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I Introduction

In presenting a concluding paper to the Symposium in honor of Professor Karrer s great contributions to organic chemistry and biochemistry, I propose to attempt an appraisal of the present state of a few aspects of work on vitamin A and to add to it a commentary based in the main on the topics discussed at this symposium

I first became interested in vitamin A in 1926 or 1927 and having watched the subject grow I am well able to appreciate our debt to Dr T Moore, whose treatise on vitamin A (1957) reduces to order the scattered and voluminous literature and shows that this one compound, placed in its

proper chemical and biological setting, gives us a glimpse of that unity of the sciences which is both a goal and a reward of research In what follows I shall try to illustrate how advances have been made and to recapture a little of the stimulus each new departure gave to those working at the subject

II A Backward Glance

Night blindness and xerophthalmia have been known since ancient times, and the special therapeutic value of liver is traditional The present century has witnessed much of the pioneer work in vitamin studies and in the course of time the vitamin A story has acquired a misleading simplicity and directness The shedding of "detail" that does not quite fit an accepted pattern no doubt helps to clarify exposition, but repeated scrutiny of the discrepancies is also necessary

In retrospect, we owe a great debt to the long line of workers who improved the rat biológical assay technique Tedious collaborative studies made clear the merits and statistical limitations of the "appeal to the animal" Comparative studies similarly enabled spectrophotometric methods to be tested, improved, corrected, and standardized The evaluation of fish liver oils and concentrates was important for research, for commerce, and for safeguarding the users of vitamin A The result was a very considerable collective achievement, the history of which would be worth writing Here, however, only a few comments are called for

1 Cod Liver Oil and the Carr Price Color Test

a Color Tests for Vitamin A Early bioassays verified that the vitamin A of cod liver oil accumulates in the unsaponifiable fraction from which most of the sterol can be removed by crystallization from methanol Rosenheim and Drummond (1925) observed a brilliant blue color when arsenic trichloride was added to a liver oil or concentrate and suspected that its intensity varied with the vitamin content This was a real discovery, but the reagent was unpleasant and dangerous, and a further step forward was taken when Carr and Price (1926) showed that a saturated solution of anhydrous antimony trichloride in chloroform was better and safer Even so it was by no means an "ordinary" color test The color was transient and there was a change in tint during the fading Worse than this, low potency oils gave a purple color whereas higher potency oils and unsaponifiable fractions gave a bright blue color

When the blue solutions were examined by spectrophotometry a sharp absorption band at 617–620 mμ was seen, and it was convenient to make intensity readings at 618 ± 3 mμ some 15 seconds after mixing the test solution with 10 volumes of the $SbCl_3$ reagent The reaction is irreversible

for when the mixture is poured into a large volume of water, anhydrovitamin A (λ_{max} 350, 370, 392 mμ) can be observed in the chloroform soluble material. This substance itself gives the same blue color with the reagent so that the large excess of antimony trichloride must almost instantaneously dehydrate vitamin A before forming the transient blue product.

When the color test is applied to cod liver oil solutions (without saponification) the absorption peak occurs at 605 mμ with a second peak or inflexion near 573 mμ. Very fresh cod liver oils or oil obtained from fresh cod livers sealed in tins and then cooked at 100°C exhibit a very marked inhibition of the color test, usually only the 573 mμ band is seen and sometimes not even that. Inhibition of the color test can be achieved with rich oils or concentrates by the addition of a little 7 methylindole (or a related derivative) *to the test solution just before adding the reagent. Under these circumstances* the 617–620 mμ peak is greatly weakened compared with that occurring near 583 mμ. These phenomena have been neglected probably because they have not been explained. It has also been stated that the blue color owes its origin to a trace of $SbCl_5$ formed in the reagent, but not much notice has been taken of the suggestion.

During the time when Heilbron held the Chair of Organic Chemistry at Liverpool, Gillam and I collaborated with him (Heilbron *et al.*, 1931) in studying the liver oils of many species of fishes. We used the Carr Price test and also carried out spectrophotometric determinations in the ultraviolet region. It was fortunate that my laboratory (in the Department of E. C. C. Baly) possessed one of the few good visual spectrophotometers then available. Photographic spectrophotometry was ill suited to studying transient processes and the dispersion at 600–650 mμ was in any case poor. Photoelectric spectrophotometry was only in its infancy. The Hilger Nutting spectrophotometer, however, was excellent for the task.

b. Carr Price Test (693 mμ chromogen). In looking at the blue solutions we often saw selective absorption at 690–700 mμ and 635–655 mμ. The appearance, with some regularity, of a color test absorption maximum at 693 mμ indicated that vitamin A (λ_{max} 617–620 mμ) was accompanied by smaller amounts of a second chromogenic substance. The two substances were obviously distinct because mammalian liver unsaponifiable fractions were devoid of the 693 mμ chromogen. In fish liver oils derived from saltwater species, vitamin A predominated over the congener by a factor of 10–20 to 1 but at that time the two substances could not be separated.

The 693 mμ chromogen was much more prominent in the liver oils from freshwater fishes and it became possible to associate the new substance with a characteristic ultraviolet absorption differing from that of the classic vitamin A. Hence the new substance was called vitamin A (Edisbury *et al.*, 1937, Lederer and Rosanova 1937).

About that time Wald (1937a) had studied the retinas of freshwater fishes and had shown how porphyropsin was obtained instead of rhodopsin, he had also observed a 693 mμ $SbCl_3$ chromogen analogous to the 617–620 mμ chromogen of vitamin A_1 and a 705 mμ chromogen analogous to his retinene$_1$ (λ_{max} 655–660 mμ) Retinene$_1$ was found to show an ultraviolet absorption peak (in chloroform) at 385 mμ

It will be recalled that carotenes (α, β, and γ) also show a blue color with the $SbCl_3$ reagent (λ_{max} 590 mμ) Although, as befits their high degree of conjugated unsaturation, the direct visible absorption of carotenoids is very intense (ϵ_{max} approximately 100,000), the intensity of the color test is relatively low Hydroxylated carotenoids such as zeaxanthin and lutein exhibit a blue color, with peaks at 621 and 585 mμ, closely simulating the vitamin A color

We are now in a position to sum up about the color test It has the merit of sensitivity (ϵ_{max} 618 mμ, 100,000) but from the beginning it had serious defects It is not specific to vitamin A, the color fades quickly and is subject to inhibition, it is different in low potency *oils* and unsaponifiable fractions In spite of its defects the color test has played an indispensable part in the advance of knowledge concerning carotenoids, vitamins, and the retinenes Perhaps there is a lesson here to be learned about the tactics of research, but I shall not pursue the matter!

c Ultraviolet Absorption Absorption spectra studies at Liverpool began in 1910 under Baly As I recollect, it was a paper by Peacock (1926) that aroused our interest in vitamin A Peacock had studied a yellow fluorescence shown by cod liver oil under ultraviolet irradiation and had shown that both fluorescence and vitamin A activity disappeared after exposure to intense light This led us to expect that vitamin A would exhibit selective absorption in the ultraviolet region

Examination of fish liver oils showed the presence of an absorption peak at *325–330* mμ *(Morton and Heilbron, 1928) The intensity of this band* showed reasonably good correlation with chromogenic power in the $SbCl_3$ color test and with vitamin A activity within the limits of accuracy then obtained Photochemical destruction of vitamin A was achieved in quartz vessels lightly silvered on the outside The absorption spectrum of vitamin A and the transmission spectrum of silver happen to be very similar, and it was found that progressive exposure to filtered ultraviolet light progressively diminished absorption at 325 mμ as well as the intensity of blue color in the Carr Price test

At first all spectrophotometric assays were made by the photographic method, which often had a rather large experimental error on the intensity scale The advent of commercial photoelectric spectrophotometers led to improved accuracy, and correction for irrelevant absorption became feasible (Morton and Stubbs 1946, 1947a,b)

Relatively satisfactory analytical methods made it easy to carry out surveys of the vitamin A potency levels of liver oils from many species. It was found that many different types of fishes gave less liver oil than cod, but of higher potency. Similarly, mammals were found to store vitamin A (in very variable amounts) in the liver. Whale liver oil became an article of commerce, and a great deal of effort was devoted to conserving the vast quantities of vitamin A present in fresh whale liver. Kitol (see Section VIII) was a complicating factor. The solution of the analytical problem ultimately required a combination of chromatographic and spectroscopic methods.

2. *Fish Liver Oils: Survey of Species*

It was natural to concentrate attention on the oils obtained from liver, and indeed careful preservation of halibut liver followed by solvent extraction has resulted in a product of high potency. Similar studies followed all over the world wherever the larger fishes could be caught. This was at a time when the commercial synthesis of vitamin A looked to most chemists to be a rather distant goal, and natural vitamin A was a highly marketable commodity, especially when in concentrated form. A large number of species was found with quite high stores of vitamin A; a selection of the list is shown in Table I.

At Liverpool we had made studies of the distribution of vitamin A, and it was a surprise to find large quantities of vitamin A in the small intestines and pyloric ceca of many species. The "viscera" of halibut, which were thrown overboard at sea, contained as much vitamin A as the liver, which was retained (Edisbury *et al.*, 1938; Lovern and Morton, 1939; Lovern *et al.*, 1939). During the period of vitamin A shortage in wartime, large quantities of vitamin A were obtained from the somewhat perishable gut tissue and only the advent of synthetic vitamin A discouraged extension of the work later.

3. *Vitamins A_1 and A_2: Distribution*

The biological implications of the large stores of vitamin A in the lining of the gut and in the liver of many, but not all, fish species have yet to be worked out. Equally puzzling are the elasmobranch fish livers, which are sometimes rich in squalene but almost devoid of vitamin A. Far too little attention has been given to the fact that vitamin A_2 (in small but variable proportions) is present in fish liver oils of marine origin. Although the relative proportion of vitamin A_2 is in general much higher in the liver oils of freshwater fishes, vitamin A_1 is nearly always present.

A species which contains only vitamin A_2 in its liver is a rarity in many countries. Abdullah *et al.* (1954) studied Nile fishes and found vitamin A_2 in liver oils in amounts varying from 0.5 to 14% (calculated as esters). One species had both A_1 and A_2 and another *Anguilla aegyptica* had A_1 only.

This attractive investigation must be seen against a complicated background Wald's original observation has been followed by evidence that both retinene$_1$ and retinene occur in the eyes of *R pipiens* tadpoles (Peskin, 1957), but only retinene$_1$ was obtained from *Bufo marinus* larvae Kennedy (1957) found porphyropsin in *R pipiens* and Collins *et al* (1953) had already found vitamin A_2 in the newts *Triturus cristatus* and *T carnifex* The situation for *R catesbiana* could not be paralleled in *R temporaria* The larvae here are small and metamorphosis occurs earlier, so that the technical difficulties are formidable

It has been suggested (Wald) that vitamin A through porphyropsin is the source of the ancestral vertebrate visual pigment To my mind the distribution of vitamin A in marine fishes, freshwater fishes, and amphibia has so many puzzling features that it seems doubtful whether legitimate conclusions about biochemical evolution can be based on the information now available

6 Carotenoid Provitamins A Sites and Modes of Conversion

The contribution by Dr Glover to this Symposium leaves us with a feeling that still more work is needed It is also important to explore further the status of astaxanthin as a provitamin A It is not possible to feel happy about the notion that β carotene itself is chiefly important in mammals as a precursor of vitamin A If we accept the idea, implicit in several of the papers read here, that vitamin A is itself a precursor of an active substance, the question must arise whether vitamin A is an *obligatory* intermediate

III Transport of Vitamin A

From the work of Ganguly *et al* (1952) much of the free (alcohol) vitamin A in plasma (cow, pig hen) was shown to be present in the proteins precipitated by half saturation with ammonium sulfate The vitamin A esters occurred with proteins precipitated at lower salt concentrations Krinsky *et al* (1956, 1958) by ultracentrifugation of human plasma came to the conclusion that, although vitamin A alcohol occurred in a protein complex, the carrier was not albumin

Garbers *et al* (1958, 1960) have used preparative electrophoresis and (2 C^{14}) vitamin A to study the transportation of vitamin A in rat serum Male albino rats exhibiting signs of vitamin A deficiency were given five daily doses of 100 I U of labeled vitamin A in peanut (*Arachis hypogaea*) oil (0 2 ml) by stomach tube Another group, beginning to lose weight on a deficient diet, was given twice weekly 175 I U labeled vitamin A for 6 weeks, so as to permit the attainment of a normal blood level and very small liver storage It was found that vitamin A alcohol is carried by a globulin

component of the serum protein, as in man In the rat the vitamin A is associated with the α_1 globulin but probably not the α_1 glycoprotein On the other hand, the esterified vitamin A is carried by the low density lipoprotein The vitamin A of rat serum (determined by the $SbCl_3$ color test) failed to account for the radioactivity in the serum after oral dosage of depleted animals The unidentified component(s) were equivalent (in terms of radioactivity) to 20–30 I U of vitamin A per 100 ml of serum, moreover they did not seem to be transported in the same manner as vitamin A alcohol

Garbers *et al* (1960) state clearly that in their work the second labeled component(s) in the rat serum may be merely a breakdown product(s) of vitamin A and not an active intermediate product(s) of vitamin A " They recall the observations of Ames *et al* (1955a) to the effect that "compounds similar in structure to vitamin A are absorbed, transported in the blood and stored in the liver " Garbers *et al* (1960) argue that as the synthetic vitamin A was labeled at C 2 "the second labeled component(s) in the serum must be expected to be relatively large or to be covalently linked to the proteins, otherwise it (they) should separate during dialysis ' The second component is not easily extracted from the serum nor can it be separated by dialysis, it is associated mostly with the α globulin

Garbers *et al* (1960) confirmed the finding of Wolf *et al* (1957) that 7–12% of the radioactivity of an oral dose of 2 C^{14} labeled vitamin A is excreted *in the urine within 48 hours*

IV Vitamin A Deficiency in the Rat and Its Relation to Ubiquinone and Ubichromenol

Lowe *et al* (1953) found that vitamin A deficiency in rats resulted in marked changes in the adrenal glands there was also an accumulation in the liver of two substances (SA and SC) having characteristic ultraviolet absorption spectra It is a source of satisfaction that Dr Johnson has so fully confirmed the histological work of my colleague, Professor Harrison

Wiss and Gloor (this volume) have discussed this and I have only a few comments to add

Morton and Phillips (1959a) followed in the rat the course of vitamin A deficiency and confirmed a progressive rise in the liver ubiquinone which occurred from the eleventh day of deprivation onward The rise in ubichromenol content became marked only near or after the time the animals reached a weight plateau Vitamin K deficiency, superimposed on vitamin A deficiency, did not appreciably affect the liver concentrations of cholesterol ubiquinone or ubichromenol (Morton and Phillips, 1959b) The rises in ubiquinone and ubichromenol that occur in the livers of vitamin A deficient rats are hastened if the rats are adrenalectomized at an early stage in the deficiency If, however, the deficiency was already advanced ad

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IV Vitamin A Deficiency in the Rat and Its Relation to Ubiquinone and Ubichromenol

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the nerve broke at the point of kinking with capping of the divided ends, which were joined only by connective tissue It appears that the actual break in the nerve may be delayed until "growth, under normal nutritional conditions has imposed a further stress on the primary anatomical lesion " It seems clear that vitamin A deficiency does result in nerve lesions which may be primary and irreversible The issues concerning bone are still somewhat uncertain

2 Hydrocephalus and Congenital Abnormalities

The work of Millen *et al* (1953, 1954) recorded that rabbits born from mothers kept on a vitamin A deficient diet often exhibited hydrocephalus Ossification was incomplete, and no bony overgrowths were seen The expanding brain under increased cerebrospinal fluid pressure brought about bone distortion, which would here be quite a secondary phenomenon The primary effect was thought to be excessive production of cerebrospinal fluid This notion is itself a little difficult since the fluid is continually produced and is normally balanced by absorption The fundamental lesion is still elusive The effects of vitamin A deficiency on pregnancy (Section VI) and embryonic development are very serious (Mason, 1935, Newton, 1938, Hale, 1933, 1935, 1937, Anderson, 1941, 1949 Warkany and Schraffenberger, 1944, 1946) Woollam and Millen (1956) include the production of diverse major congenital abnormalities in the heart, kidneys, genitourinary tract, eyes, ears, palate, and at other sites in the body in many species, and anophthalmos can occur in pigs Vitamin A deficiency makes its greatest impact at the particular stage in gestation when the supply to the fetus falls below some critical level and some crucial process fails to proceed normally for want of the appropriate biocatalyst This crucial process, if it exists, must have a high degree of generality for growth processes In our work on vitamin A deficiency in the domestic fowl (Lowe *et al*, 1957), there was marked retardation of sexual development in males and none of the vitamin A deficient pullets laid any eggs

The over all picture of the damage done by vitamin A deficiency inspires awe Dr Moore says in his book "It seems reasonable to conclude that vitamin A deficiency can have *primary* effects on nerves, bones and epithelial tissues " One can agree that the attempt to sort our primary and secondary effects has not been wholly successful It is equally necessary to say that not one of these effects has yet been related to the chemical structure of vitamin A in a scientifically serviceable manner

VI Vitamin A and Pregnancy

Lund and Kimble (1943) observed that in pregnant women plasma vitamin A was lower in the third trimester than in the second but that it quickly

rose within 48 hours of parturition Bodansky *et al* (1943) made similar observations and agreed that there was no corresponding fall in the carotenoids of plasma The differences in vitamin A levels were not great, thus for 70 women pregnant for less than 6 months, the average was 105 I U / 100 ml, and for 62 women pregnant for more than 6 months the average was 91 I U /100 ml plasma Again 26 women given 10,000 I U of vitamin A per day showed on the average 123 I U /100 ml whereas 27 undosed pregnant control" subjects had 93 I U /100 ml plasma on the average

Sutton *et al* (1945) studied 28 cows before and after parturition Plasma vitamin A levels (averaged) fell from about 74 to 35 I U /100 ml during the 3 weeks preceding parturition, then remained low for a week post partum and afterward rose quickly Plasma carotene similarly fell from over 600 µg /100 ml to under 400 and then rose rather slowly

Goodwin and Wilson (1951) confirmed the main findings but thought that the changes were part of a more far reaching metabolic display at or near parturition The temporary fall may well result in part from the accumulation of vitamin A in the colostrum which is being formed in the mammary glands Cow colostra with 600–900 I U /100 ml of vitamin A and 200–500 µg of carotenoids per 100 ml are several times richer in both components than the later milk This applies also to other species

Thompson and McGillivray (1957) studied cattle and heifers in New Zealand, where pasture feeding ensures a high carotene intake round the year In spite of this, plasma levels of vitamin A and carotenoids were found to decrease at or near to parturition Vitamin A alcohol fell from 90 I U to 53 3 I U /100 ml just after parturition and then rose until after 11 days a level of 80 I U /100 ml had been regained The carotene level rose to about 1350 µg /100 ml just before parturition and then fell abruptly to about 900 µg /100 ml and further to less than 750 µg /100 ml on the third day Thereafter the carotene levels rose again

Vitamin A ester, almost 5 µg /100 ml one or two days before parturition, fell to about 3 µg /100 ml from one to three days after parturition Thompson and McGillivray interpreted the evidence as supporting the suggestion 'that the decrease in plasma components is related to the extra drain of colostrum synthesis rather than to any hormonal changes associated with parturition '

Lubke and Finkbeiner (1958) studied the serum levels of carotene and vitamin A in human pregnancy, at parturition and puerperium This is an important investigation on a firm statistical basis

Figure 1 illustrates the results The mean serum vitamin A for healthy nonpregnant women (193 ± 24 I U /100 ml) fell abruptly (to 143 ± 28 3 I U /100 ml) at the onset of pregnancy and then slowly rose (to 166 ± 27 5) just before parturition There was then a further sharp fall (to 125 3

± 32 3 I U /100 ml) The level in cord blood was only about half that in maternal circulation During the first few days after parturition the serum level rose considerably (to over 220 I U /100 ml) and thereafter fell to nearly the average figure for nonpregnant women The figure also shows how the β carotene level of serum also fell strikingly in the early period

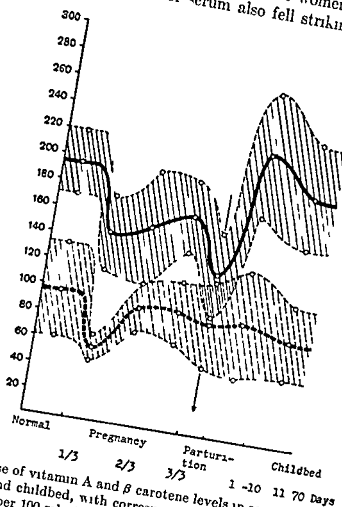


FIG 1 Course of vitamin A and β carotene levels in serum during pregnancy and in parturition and childbed, with corresponding standard deviations Upper curve vitamin A I U per 100 ml lower curve β carotene, micrograms per 100 ml After Lubke and Finkbeiner (1958)

of pregnancy but rose again during the second half to regain the initial level The striking fall in vitamin A at parturition was not paralleled The carotene level in cord blood was about 25 % of that characteristic of maternal blood

In a proportion of their cases, Lubke and Finkbeiner observed the presence in serum of unusual substances (possibly breakdown products of carotenoids) that interfered with spectroscopic assays They were seen only during pregnancy and at parturition or during the first weeks post partum These substances, about which little more was said, deserve further study

The fetus is well protected against excess of vitamin A or carotenoid Only a small proportion of either finds its way across the placental barrier, and heavy dosing has but little effect Liver reserves of the newborn are quite low in most of the species that have been studied *Per contra* the colostra, rich in vitamin A and often rich in carotene, are well absorbed and so help to build up a store in the liver of the offspring

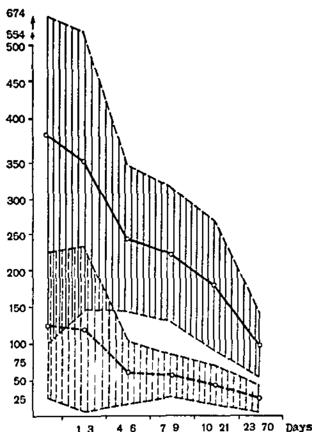


FIG 2 Colostrum and milk decrease of vitamin A and β carotene values in mothers milk Upper curve vitamin A I U per 100 ml lower curve β carotene micrograms per 100 ml

It is quite clear, however, that the vitamin A and carotene levels of milk soon fall [Fig 2, after Lubke and Finkbeiner (1958)] Lubke and Finkbeiner (1958) write ' Schwerwiegende Folgen aus einer unzureichenden Versorgung mit Vitamin A im intrauterinen Leben und auch im Sauglingsalter sind bekannt Die Seltenheit des Auftretens von manifesten Vitamin A—Mangelerscheinungen darf jedoch nicht dazu verleiten die Bedeutung des Vitamin A zu unterschatzen " They recall a suggestion by O Neumann (1950) that vitamin A acts as a catalyst for the transformation of cholesterol into progesterone The implication here is that at the onset of pregnancy

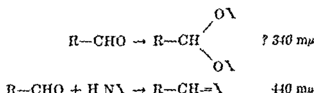
there is a sharply increased demand for vitamin A at some site or sites in the body and the rate of withdrawal of the vitamin from the blood stream temporarily exceeds the rate of mobilization of vitamin A from the liver reserve There is no evidence that in these women the blood levels of vitamin A became dangerously low or that their liver reserves were depleted

It is, however, important to consider what might have happened if the reserves had been low initially There is also the rather uncomfortable feeling that magnitude of the normal liver reserves may sometimes lead to an illusory sense of security if they are not readily mobilized in physiological emergencies

VII Visual Pigments

At this stage I have little to add to the recent brilliant work of Wald and his school on the role of *cis trans* isomerism (Wald, 1952, 1958) The elucidation of the relationship between one *cis* isomer and opsin to form rhodopsin leaves at least two major questions unanswered First, there is no satisfactory explanation in terms of chromophoric groups of the plurality of visual pigments, nor for the shifts rhodopsin (500 mμ) → porphyropsin (522 mμ), iodopsin (560 mμ) → cyanopsin (620 mμ)

The attractive work of Goldsmith (1958) on the visual system of the bee (*Apis mellifera*) by the recognition of retinene in the head tissue suggests that insect vision may have much in common with that of other animals Yet the photosensitive substance has λ_{max} 440 mμ and is readily soluble in plain water Further light sensitive materials have sensitivity maxima at 335–340 and 490 mμ To get from retinene$_1$ with λ_{max} 370 mμ to a substance with λ_{max} 335–340 mμ, it is necessary to neutralize the C=O chromophoric effect without producing the bathochromic shift seen in a Schiff base, e g



1 Action of Enzymes on Rhodopsin

Radding and Wald (1958) showed that chymotrypsin attacks rhodopsin in two stages (1) an initial rapid process exposing 30 amino groups per molecule without bleaching and (2) a slower hydrolysis exposing about 50 additional amino groups *with proportionate bleaching* Chymotrypsin attacks peptide linkages of aromatic amino acids with methionine, apparently in rhodopsin itself rather than in protein impurities This seems to indicate fragmentation of rhodopsin without attack on the site of attachment of

retinene since there is no "bleaching" The chymotrypsin was a purified, crystalline specimen The slow process is accompanied by a steady fall in absorption at 500 mμ

Now the essence of much of Wald's work is that opsin the intact protein can unite with retinene$_1$ to form regenerated rhodopsin Whatever the nature of the link between the aldehyde group and the protein it cannot be a peptide link of the kind postulated as the locus of chymotrypsin action Radding and Wald say 'it may be concluded that the hydrolysis and bleaching of rhodopsin observed in this experiment resulted from the enzymatic action of chymotrypsin If this is the case we must interpret the enzymatic action to *include* processes brought about by the *products* of digestion The pH changed from 7 82 to 7 21 during the hydrolysis and this may have caused a nonenzymatic fission of retinene from rhodopsin

Purified pepsin and trypsin each hydrolyzed and 'bleached rhodopsin but pancreatic lipase was ineffective Pepsin and chymotrypsin (but not trypsin) show a marked preference for links involving aromatic amino acid residues The effectiveness of all three enzymes leaves us very much in the dark about the relationship between bleaching and enzymatic hydrolysis

It seems unlikely that all three enzymes will attack the —CH=N linkage which is perhaps the basis of the retinene attachment Radding and Wald were themselves uneasy, for they wrote 'alternatively, it may be that the initial fragmentation leaves the chromophoric site unstable so that the fragments bleach spontaneously without further hydrolysis [presumably without further enzymatic hydrolysis] In that case the proportionality between bleaching and hydrolysis in the slow phase of the reaction is fortuitous

It is known that phosphopyridoxal can combine unspecifically with amino acids in the absence of enzymes to form Schiff bases The combination of retinene with amino compounds is similarly unspecific when the product resembles indicator yellow (Lythgoe 1940), but to form a pigment with λ_{max} 500 mμ (rhodopsin) or 560 mμ (iodopsin) requires something more about which we know little If as seems possible, retinene is *doubly* attached to opsin, much remains to be done before these actions of pepsin and chymotrypsin can be properly understood

2 Cis trans Isomers of Vitamin A and Retinene$_1$

If we turn to the results of biological assays on rats we find the results in Table II The aldehydes of the 2 mono *cis* and 2 4 di *cis* types are more active than the corresponding vitamin A alcohols possibly because the former are more readily isomerized *in vivo* to the all *trans* form In view of the fact that neovitamin A and all *trans* vitamin A are interconvertible in the animal body so that the all *trans* form becomes greatly predominant

(Robeson and Baxter, 1947), there is no compelling reason to suppose the neovitamin A is *at all active per se* The principle of Occam's razor applied to Table II implies that so far as the neo and 2,4 di *cis* isomers are concerned there is no need to postulate that they are intrinsically active The suggestion is not quite so plausible for the 6 mono *cis* and 2,6 di *cis* isomers, but there is even so no evidence in favor of the idea that they are active per se All that we can assert with confidence is that, on the basis that the animal body stores both all *trans* and neovitamin A, either could be the precursor of whatever active form or "coenzyme" actually participates in metabolism

TABLE II
RELATIVE BIOLOGICAL ACTIVITIES

	Vitamin A isomers [b]	$Retinene_1$ isomers [c]
All *trans*	100	91
2 Mono *cis* (*neo*)	75	93
6-Mono *cis*	22	19
2 6 Di *cis*	24	17
2 4 Di *cis*	23	48

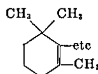
[a] As acetates growth test in rats
[b] S R Ames *et al* (1955a)
[c] S R Ames *et al* (1955b)

VIII Vitamin A Acid, Vitamin A and Kitol

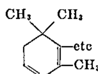
The case of vitamin A acid is especially interesting The elegant work of *Arens and van Dorp* (1946) *showed that the sodium salt*, given by mouth, had the full activity of vitamin A alcohol but that no vitamin appeared in the liver even when high doses of the sodium salt were given Sharman (1949) confirmed this, and Wald and his colleague Dowling have proved clearly that although vitamin A acid is useless so far as vision is concerned it works well in the rest of the body The methyl ether of vitaminA_1 has been synthesized by Isler *et al* (1946) and prepared from natural vitamin A by Hanze *et al* (1946) This compound is very active by the rat growth test, but the butyl and phenyl ethers are not There are two possible ways to look at this one is to suppose that the methyl ether readily loses CH_3 *in vivo* to yield vitamin A alcohol, and the other is to suppose that the terminal part of the molecule is not concerned in the systemic mode of action There is too little clear cut evidence to permit a choice between these alternatives

The most important ... vitamin A fr... point of view is that the ring ... om that of vi... all appear

ances retinene$_2$ is quite as efficient (in appropriate species) in providing visual pigments, and in some fishes that have no vitamin A_1, vitamin A_2



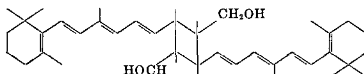
Vitamin A_1



Vitamin A_2

serves all the systemic purposes of vitamin A_1 (Balasundaran *et al*, 1956) This does not permit us to *deny* that the β ionone ring—with or without the second double bond—is the locus of chemical change associated with biological function but it does permit us to assert that neither is *specific*

Kitol is best known as a (relatively) major constituent of whale liver oil (Pritchard, *et al*, 1937, Embree and Shantz, 1943 Clough *et al*, 1947) It has λ_{max} 290 mμ and much lower intensity of absorption than vitamin A this indicates a chromophoric grouping of not more than four conjugated double bonds In fact it is a divitamin A $C_{40}H_{60}O$ with eight double bonds and two hydroxyl groups It is itself biologically inactive but on pyrolysis in a high vacuum it yields vitamin A The structure is said to be



It is reasonable to think that since kitol is inactive per se and does not yield any biologically active metabolites the loss of the double bond adjacent to the primary alcohol group is highly significant

IX. Hypervitaminosis A Induced by Vitamin A Acid

Pitt and Thompson, working at Liverpool (unpublished observations) have found that in respect of the effect of vitamin A deficiency on the rat liver contents of ubiquinone (45) and ubichromenol (Section IV) vitamin A acid (sodium salt) can replace vitamin A very satisfactorily (Table III) Vitamin A acid brought about the full syndrome of hypervitaminosis in about half the time and at about half the dosage compared with vitamin A (administered as high potency fish liver oil) When 1 month old rats were given 7 5 mg vitamin A per day as fish oil they lived for 3–4 weeks, but when vitamin A acid was given at that dose level the animals were severely ill within 48 hours, and even 1 mg per day produced hemorrhages and shortened life

This greater toxicity of vitamin A acid is not inconsistent with the idea (Moore, Wald, Johnson) that vitamin A alcohol is a precursor of "active

vitamin A which the body does not need to store This active form could be the acid, or something derived from it

X Hypervitaminosis A in Humans Induced by Vitamin A Alcohol

When considering the fate of vitamin A we can look at the matter from two different standpoints

1 In the healthy adult the maintenance requirement is probably between 1250 and 2500 I U per day or roughly 0 4–0 8 mg per day Assuming that vitamin A (or something derived from it) has catalytic functions, this requirement is to replace *leakage* from cyclical operations It is reasonable

TABLE III

Comparison of Vitamin A Alcohol and Vitamin A Acid in Counteracting the Rise in Liver Ubiquinone in Rats on a Vitamin A deficient Diet[a]

Group	Diet	Ubiquinone in liver	
		µmoles/gm	µmoles/liver
I	Vitamin A deficient[b]	0 446	1 212
II	Vitamin A deficient plus vitamin A alcohol 30 µg /day	0 172	0 879
III	Vitamin A deficient plus vitamin A acid (sodium salt) 100 µg /day	0 150	0 745

[a] G A J Pitt and J N Thompson unpublished data
[b] Amount of food not restricted
[c] Amount of food restricted to that eaten by Group I

to suppose that the irreversible loss will be subsequent to the conversion of vitamin A alcohol or acid to the "active" form and that the degradative pathway will make use of pre existing mechanisms

2 An adult undergoing vitamin A therapy may ingest 2000 I U per kilogram body weight or about 36–42 mg per day Something like one third of the dose (12 14 mg per day) may be absorbed and most of it will be firmly "tucked away" in the Kupffer cells of the liver It does not necessarily follow that the excess vitamin A which may transiently appear in the blood plasma will be eliminated by the normal "leakage" pathway Josephs (1944) reported on a 3 year old child who had been given about 240 000 I U per day in the form of halibut oil since he was 3 months old, i e about 80 gm vitamin A Plasma vitamin A at 900 I U /100 ml was high and the child showed typical hypervitaminosis A Plasma levels fell to 650 I U /100 ml after 20 days, 280 I U after 7 months and 40 I U /100 ml after 2½ years when the dosage was discontinued Many of the symptoms of hypervitamin

osis disappeared quickly, but hypertrophy of liver and spleen lasted longer, as did the skeletal abnormalities In other cases very high dosage did not raise the plasma vitamin A level and no signs of hypervitaminosis appeared In these cases the liver seemed capable of removing from the blood stream the ingested and absorbed vitamin and locking it up in the Kupffer cells

From this we can conclude *either* that vitamin A is itself toxic to certain tissues or that a degradation product accumulates in undesirable amounts when the plasma level is unduly high It seems very difficult to believe that vitamin A is in itself a general tissue poison for the following reasons

1 Many species of fish exhibit vast quantities of vitamin A in the lining of the intestines as well as in the liver These intestinal mucosae are the sites of so many different metabolic processes that if vitamin A were an enzyme inhibitor it might be expected to be toxic

2 Some species have very appreciable amounts of vitamin A in heart tissue—again a site of diverse metabolic processes

3 Under certain circumstances kidney tissue becomes relatively rich in vitamin A This is normally the case for cats

4 There is no evidence that the liver vitamin A inhibits metabolic processes or that in the choroid layer of the eye the vitamin is in any sense toxic

If then the notion that vitamin A *itself* is directly toxic has little to recommend it the phenomena of hypervitaminosis A must be due to a derived substance or substances

XI VITAMIN A ALDEHYDE (IN COMBINED FORM) IN EGGS

The Reading group led by Kon has made notable contributions to the study of vitamin A in invertebrates (for a review see Fisher and Kon, 1959) The Crustacea that contain vitamin A have most of it in their eyes, where it exists as ester to the extent of 90% mainly as the 11 *cis* isomer The amount is much in excess of the retinene present in rhodopsin, and it is evident that the animals' reserves are stored in the eyes Invertebrates such as cephalopods and euphausiids provide a source of vitamin A for their predators Indeed the euphausiids are regarded as the base of vitamin A pyramid in marine economy The vitamin A of the euphausiids is possibly derived from the astaxanthin of copepods (Grangaud and Massonet, 1955, Fisher *et al*, 1954) This is a plausible explanation of the high vitamin content of *Meganyctiphanes norvegica* (M Sars), on which many fishes feed

Plack *et al* (1957 1959) made a very interesting discovery when they found (combined) vitamin A aldehyde in fractions obtained from herring ovaries (*Clupea harengus* L) The background of this work is worth noting Several workers (Scheunert and Schieblich 1934, Junker, 1952–1953 1956) had found substantial' amounts of vitamin A in hard roe by biological

assays whereas the amounts of vitamin A ester and alcohol or carotenoid provitamins A (Fisher *et al*, 1952, Thompson *et al*, 1949) found chemically accounted for only one tenth of the biological activity Kon was justified in reminding us that "if we are not to miss the wood for the trees, we should always have at hand the means of appeal to the living organism as a check on the dumb efficiency of our instruments "

When the investigation was reopened it was observed that 70% of the activity could be extracted by light petroleum in the presence of ethanol From the extract, vitamin A_1 aldehyde varying in amounts between 2 4 and 5 7 μg per gram of eggs was obtained, together with smaller amounts of free and esterified vitamin A The eggs of seven other species of marine teleost fishes contained vitamin A aldehyde (1–3 μg /gm) and vitamin A (0 1–0 3 μg /gm)

Plack *et al* (1959) emphasize a technical point of some significance If ethanol is added to the eggs, "the protein on the outside of the eggs" is denatured 'to produce hard spheres not easily broken down " For this reason the eggs are first homogenized alone, then with petrol, before the ethanol is added Even so, 30% of the apparent biological activity has not been accounted for (Treatment with potassium hydroxide or hydrochloric acid would have caused losses of aldehyde) Plack (1960) has been able to show that hens' eggs contain vitamin A_1 aldehyde bound to a lipid and (directly or indirectly) to protein [Vitamin A (free) 100 μg , (esterified) 19 μg vitamin A aldehyde 22 μg , per egg]

The material *actually* extracted from herring eggs is distinctly different from vitamin A aldehyde With the $SbCl_3$ reagent it gives a "weak blue grey colour" quite different from that shown by the aldehyde (λ_{max} 664 mμ) Only after chromatography on "water weakened alumina" is the aldehyde itself obtained The absorption curves of the petrol extracts show peaks at 325 mμ and 360 mμ, indicating that most of the "aldehyde" is combined through the CHO group in a manner that neutralizes the chromophoric effect of the conjugated carbonyl group Plack *et al* (1959) go on to say "Assuming a molecular weight for the complex twice that of vitamin A aldehyde, the active material comprises only 1/5,000 of the weight of the lipid present " Another puzzling phenomenon is that the effect of acetone on the extract of herring eggs is to reduce the biological activity to 40% of the original

One uncomfortable outcome of this work is that statements that vitamin A is absent from a species or a tissue must be re examined Nonsaponifiable fractions will contain vitamin A itself, which can be detected, but traces of aldehyde (or related compounds) present in natural products may easily have escaped detection

XII Modes of Action

Some vitamins, such as pantothenic acid and p aminobenzoic acid, occur as mid portions of more complicated functional entities Thus in coenzyme A the amino group of pantothenic acid is in peptide linkage and the primary alcohol hydroxyl is attached to a pyrophosphate grouping In folic acid the amino group of p aminobenzoic acid forms the link with a pterin moiety and the carboxyl group joins the glutamic acid residue Neither in the case of coenzyme A nor that of folic acid would a study of the chemistry of the mid portion have led to serviceable hypotheses concerning biological roles Even the part played by N^{10} formyl tetrahydrofolic acid, where the nitrogen of the p aminobenzoic moiety is the locus of transfer, could not be foreseen and had to be discovered by experiment

If the systemic mode of action of vitamin A is akin to that of pantothenic acid or p aminobenzoic acid i e if vitamin A is a mid portion of a larger molecule, we are chasing a will o' the wisp in seeking a direct explanation

On the structural side it is at least possible to clear the ground

1 The unsaturated chain is indispensable because dehydrovitamin A (Gould 1936) tetrahydrovitamin A (Ruzicka and Fischer, 1934) and perhydrovitamin A (Karrer and Morf 1933) are all devoid of biological activity

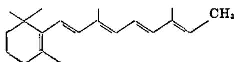
2 Since vitamin A acid and vitamin A methyl ether as well as vitamin A alcohol can have very high activity the primary alcohol group is not indispensable Axerophthene ($C_{20}H_{30}$) has about one fifth the potency of vitamin A (von Euler and Karrer, 1949) Vitamin A acid is metabolized so much more quickly than vitamin A alcohol that it might be more closely related to the active form

3 Since vitamin A_1 and A_2 are in appropriate species, fully effective, neither the β ionone ring nor the dehydro β ionone ring is fully specific to the action

4 Vitamin A cannot be found in many tissues where the case for its necessity is very strong

5 There have been numerous references to possible hidden" forms of vitamin A, among the more recent ones being the masked aldehyde of Kon's group and the unusual compounds noted by Lubke and Finkbeiner (1958) in pregnancy serum and the new material of Garbers *et al* (1958, 1960) and Lowe and Morton (1956)

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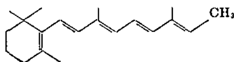
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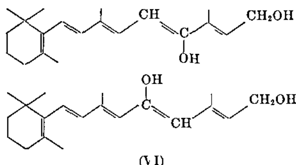
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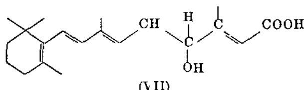
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(VI)

If we follow out the idea that vitamin A acid is nearer the active form it is conceivable that (VII) may be a link



(VII)

This is only "paper" chemistry and its justification rests solely on the fact that we have no good antivitamin A and do not know how vitamin A works

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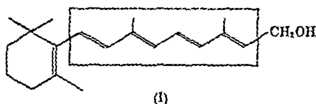
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had one fifth the potency of vitamin A itself, and the suggestion was made that a little of this substance is made *in vivo* when anhydrovitamin A is tested

7 If the *active* form of vitamin A is to participate in dehydrogenase systems and/or enter into reaction with the sulfhydryl groups of proteins or with calcium ions, it must have some new "aspect"

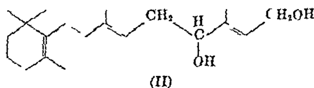
8 Furthermore, all that we know suggests that it will itself be rapidly metabolized

I am therefore led to look at vitamin A_1 or A with my attention focused on the portion of the molecule (I) enclosed in a rectangle

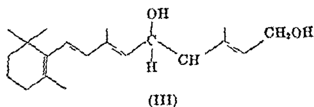


(I)

Dr Isler invited us to suggest compound which it might be worth while to synthesize Greatly daring may I speculate about a hypothetical hydrase leading to (II) or (III)

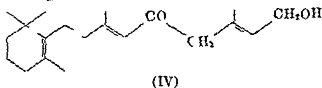


(II)

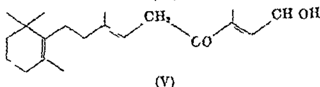


(III)

A dehydrogenase might then form one (or two) ketones (IV, V)



(IV)



(V)

These compounds, if they did not enolize, would be spectroscopically distinguishable In the presence of alkali they might enolize to give (VI)

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Excerpts from the Concluding Remarks

P KARRER

We have arrived at the end of the symposium on vitamin A, and it is a pleasant duty for me to express some further words of thanks before we separate

Above all I thank all the colleagues who have contributed by a communication to the success of the meeting and I also thank all those who animated the symposium and made it fruitful by their discussions

The papers and discussions of these past days have shown how many problems are still open in the field of vitamin A and how difficult it is to interpret many observations concerning the biological activity of this vitamin

Colleagues from seven different countries took part in this symposium—fresh proof of the international character of science Some years ago on a similar occasion I referred to the words of Goethe 'Science is a great musical fugue, in which the voice of every nation finds its expression" and I added "This metaphor contains a deep truth" When anywhere in the world a scientist raises his voice to pronounce a new thought, he is answered next day by a colleague in another country and the day after a third voice joins in from another part of the world, and soon we hear a concert that extends over the whole earth Is there anything better than these free discussions from country to country, from continent to continent which do not serve to subject humanity nor to impose power but which are aimed at unveiling the secrets of nature and extending our understanding of the universe? We must hope that this influence of science, which it shares with art, may affect all spheres of human activity Science has been compared to a lever with which the world can be lifted But let us be modest, let us not forget that science is not the *only* lever, and that the spoken word ideologies and religions have shaken and changed the world just as much as has science We scientists must never believe that we alone know and possess the truth we must be humble and admit that science is only one of the factors that determine man and the soul of man

But let us cherish science, and continue to hope that the results of science will contribute something to make human life more agreeable and more beautiful

With these words I conclude our vitamin A symposium and wish you all a happy return home

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